STUDIES ON THE VIRUS OF LOUPING-ILL (*ERRO SCOTICUS*)

by

Holman Eugene Williams, D.V.M., M.Sc.

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Acknowledgement

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INTRODUCTION

The identification of louping-ill (Erro scoticus) infection in animals is invariably dependent on the application of the serum protection or neutralisation test in a susceptible test animal. Alston and Gibson (1931) reported the susceptibility of the mouse to louping-ill and this species has remained the animal of choice for the titration of virus and antibody; both the intracranial (Schwentker et al., 1933) and the intraperitoneal routes (Pond et al., 1953) have been used in this regard.

Multiplication of the virus of louping-ill in the embryonated hen's egg, and the development of pocks on the chorio-allantoic membrane, were reported by Burnet (1936); however, chorio-allantoic titration of virus was unsatisfactory, due to the occurrence of non-specific lesions. Wilson (1944) showed that louping-ill immune serum could be titrated both by its inhibitory action on the growth of virus in tissue culture and by neutralisation in vivo where serum was injected on one side of the sheep and virus on the other. The former technique required the addition of decreasing quantities of immune serum and a constant amount of virus to a series of freshly prepared Maitland-type culture systems. After suitable incubation, the presence or absence of virus was detected by the intracerebral inoculation of culture fluid into mice. Edward (1947) found that the fatal infection/

infection of the chick embryo with louping-ill inoculated into the yolk sac afforded a means of titrating virus which was about one hundred times more sensitive than intracerebral titration in mice; the method was also successfully used for serum neutralisation tests.

Casals and Palacios (1941) reported the development of a complement-fixation test in louping-ill using antigen prepared from infected mouse brain and hyperimmune mouse and monkey sera and also human convalescent sera. The antigen was an aqueous brain suspension repeatedly frozen and thawed and then centrifuged at 3,500 R.P.M. for 1 hour; later (Casals, 1947) this was increased to 7,000 R.P.M. for the same period. The heating of sera to various temperatures, depending on the species, was a necessary feature for the success of the test.

The application of the complement-fixation test to the disease in domestic animals has been very limited. An unspecified complement-fixing antigen was used by Edward (1948) to measure antibody induced in cattle by various vaccines. The results were equivocal. The same worker (1950), however, using the method described by Casals and Palacios (1941), was able to show complement-fixing antibody in the sera of two hyperimmune sheep. One animal, with a neutralisation index of 300, gave a titre of 1 in 16 while the other titred at 1 in 64; the lowest dilution of serum tested was 1 in 2. The strongly anti-complementary nature of bovine serum precluded Dunn (1952) from using the/

the test in diagnosing the disease in cattle. Neither Dunn nor Casals and Brown (1954) were able to demonstrate haemagglutinins for the virus of louping-ill.

Although louping-ill in sheep is frequently associated with a varying degree of destruction of the Purkinje cells of the cerebellum (Brownlee and Wilson, 1932), the histological diagnosis of the disease is unacceptable. Apart from the complement-fixation test, therefore, all other means of identifying the disease are dependent, at some stage or other, on inoculation into a susceptible test animal or into the embryonated egg. The object of the following investigation was to attempt to develop a suitable in vitro procedure for identifying the virus of louping-ill with a view to obviating costly and time consuming animal titration. Section one deals with the application of the complement-fixation test in the diagnosis of the disease in sheep and its usefulness as a measure of immunity in this species. Section two is concerned with the role of tissue culture as a direct means of diagnosing the disease.

I. THE COMPLEMENT-FIXATION TEST IN SHEEP

INTRODUCTION

It is well known that many viruses fix complement in the presence of their immune sera. Notable among these are vaccinia (Gordon, 1925; Bedson and Bland, 1929), foot-and-mouth disease virus (Ciuca, 1929), herpes (Bedson and Bland, 1929), and influenza (Smith, 1936). Antigen was invariably prepared from tissue abundant in virus. Two sources of virus were selected in regard to louping-ill, namely, infected mouse brain and fluid of tissue culture in which virus had been propagated.

Complement-fixing antigen prepared from brain tissue has been studied in a number of neurotropic diseases. Such antigen possesses several disadvantages. Extraneous tissue may be anti-complementary and/or may combine with test sera to react non-specifically. Investigating the latter reaction, Kidd and Friedwald (1941) concluded that a natural antibody of normal serum reacting with a sedimentable constituent of normal tissue was in part responsible. Brain tissue may contain relatively low amounts of specific complement-fixing substances. Antigen tends frequently to become anti-complementary on storage for short periods.

Several procedures have been employed with a view to overcoming these difficulties. Utilisation of brain antigen in a high dilution was the approach of Havens and Mayfield (1932) who/

who reported specific fixation between the rabies virus in rabbit brain and immune guinea-pig serum. The antigen was a 1 per cent. tissue suspension and antigenicity was of a low order. Also working with rabies virus in sheep brain, Greval (1933) relied on large quantities of complement (8 to 10 M.H.D.) to suppress the anti-complementary effects of the various constituents in the test. Howitt (1937), using a method based on that of Craigie and Tulloch (1931), dried brain antigens infected with equine encephalomyelitis, lymphocytic choriomeningitis, and St. Louis viruses from the frozen state; the dessicated tissues were then extracted with ether to remove the anti-complementary lipid fractions. The antigens were used as 1 per cent. suspensions and although specific fixation was demonstrated the margin between specificity and non-specificity was narrow.

Casals and Palacios (1941) were able to prepare specific brain antigens for louping-ill and several other neurotropic diseases by ultracentrifugation or filtration through a Seitz pad. Specific antigenicity, however, was reduced to a variable degree by the latter treatment. In order to avoid ultracentrifugation, these workers advocated antigen prepared by repeatedly freezing and thawing the suspension of infected tissue. This step was included not so much to release intracellular virus but to produce a flocculate which was readily removed by moderate centrifugation. They also found that non-specific reactions which occurred between normal and immune sera and brain tissue could be eliminated by heating the sera to various temperatures/

temperatures depending on the species concerned. Heat also destroyed any anti-complementary effects of sera which sometimes occurred even in the absence of antigen.

Havens et al. (1943), employing differential centrifugation, were successful in the preparation of mouse brain antigens for St. Louis, Japanese B, and West Nile encephalitis and eastern and western equine encephalomyelitis viruses. The final centrifugation was carried out at 12,400 R.P.M. in a Pickels machine for 1 hour in the cold. Similarly, Le Bouvier (1953), combining both freezing and thawing with ultra-centrifugation, prepared a satisfactory complement-fixing poliomyelitis antigen from infected mouseling brain. That ultracentrifugation might not be generally useful in preparing antigens, however, was suggested by Casals (1947); he found Japanese B mouse brain antigen centrifuged at 18,000 R.P.M. to be four times less active than that prepared by repeated freezing and thawing, while dilutions of mouse-immune serum yielded only half the titre with the former antigen.

More recently, brain antigen prepared from tissue dried in vacuo from the frozen state and extracted with organic solvents has again been used. In this regard, De Boer and Cox (1947) found benzene, toluene, and dichlorethylene to be superior solvents to ethyl alcohol, ethyl acetate, diethyl ether, and carbon tetrachloride in extracting mouse brain and chick embryo antigens infected with eastern/

eastern and western equine encephalomyelitis, St. Louis, and Japanese B viruses. The particular advantage of these antigens over those prepared by repeated freezing and thawing was that non-specific reactions of human syphilitic and malarial sera were abolished. These results were in general confirmed by Espana and Hammond (1948).

Preparation of antigen from mouseling brain was reported by Casals et al., (1950, 1951, 1952). Apart from the advantage in these instances of adapting poliomyelitis virus to the suckling mouse, its brain tissue possesses appreciably less lipids than that of the adult mouse and tends, therefore, to be less anti-complementary. Pollard (1951) purified the virus of poliomyelitis from infected cotton rat brain by methanol precipitation; the virus was eluted in a M/5 phosphate buffer of pH7.0. Pollard claimed that lyophilisation appeared to inactivate the virus and to destroy its antigenic properties.

The propagation of viruses in tissue culture has made antigens available which are relatively free from extraneous tissue and of increased titre in some instances. Complement-fixing antigens from culture have been prepared for a number of viruses. Smith (1936) produced a satisfactory influenza antigen from virus grown in chick embryo tissue suspended in Tyrode's solution. Svedmyr and his co-workers (1952, 1953) concentrated the crude culture fluid containing poliomyelitis virus grown in human tissues by ultrafiltration, but Enders (1955) and Baumeister and Miller (1956)/

(1956) have now grown poliomyelitis virus in sufficient titre to preclude the need for its concentration for use as antigen in complement-fixation. Similarly, complement-fixing antigens have been prepared for vesicular exanthema (McClain et al., 1954), foot-and-mouth disease (Brooksby and Wardle, 1954; Sellers, 1955), vesicular stomatitis (Sellers, 1955), and infectious canine hepatitis (Fieldsteel, 1956).

That the virus of louping-ill would grow in vitro was shown by Rivers and Ward (1932), utilising the method described by Li and Rivers (1930) and Rivers (1931) for the cultivation of neuro-vaccine virus and dermo-virus in a system consisting of triturated viable chick embryo suspended in Tyrode's solution with or without monkey serum. Wilson (1944) adopted this method for growing the virus but substituted whole embryo for chick embryo brain or embryos without brains, and normal sheep serum for monkey serum, when serum was used. Antigen prepared in this manner was of relatively high titre and proved to be a successful immunising agent in sheep. There is no record of its use as a complement-fixing antigen.

MATERIALS AND METHODS

1. Sheep sera

A. Hyperimmune sera

(i) Antigen for hyperimmunisation

The antigen employed for this purpose was viable homologous virus in brain and cord tissues recovered in the terminal stages of the experimental disease induced by intracranial inoculation of the agent. Tissues, secured in an aseptic manner from a number of infected sheep, were pooled, blended in an 'atomix', and stored in the frozen state at -28°C. in paper cups. Virus pools were titrated by inoculating mice with tenfold dilutions of tissue in a diluent of physiological saline (0.85 per cent. NaCl) containing 10 per cent. normal sheep serum; they were kept under observation for 15 days thereafter. The LD_{50} dilution was calculated by the method described by Reed and Muench (1938) and titres are expressed throughout, unless otherwise stated, as the log LD_{50} dilution when 0.05 ml. is inoculated intracerebrally into 3 to 5 week old white mice. Virus is stable in a saline-serum solution in the proportions described over a period of at least 4 hours at room temperature (22°C.) (Smith, 1956) while normal serum is regarded as that free of neutralising antibody to louping-ill. The titres of the 2 brain pools used in hyperimmunisation were $10^{-6.0}$ and $10^{-6.3}$ (table 1). Those of other organ pools from the same sheep are also shown.

Table 1

Mouse titrations of louping-ill virus in various organ pools from sheep sacrificed in the terminal stages of the disease following intracranial inoculation of virus.

Pool		Organ - log dilution							Titre*
No.	Organ	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}	
1	Brain-cord	-	-	-	7 8 8 9	8 8 10 11	8 11 S S	-	$10^{-6.0}$
	Spleen	7 7 8 S	7 12 15 S	9 S S S	S S S S	-	-	-	$10^{-2.3}$
	Liver	S S S S	S S S S	-	-	-	-	-	
	Kidney	S S S S	S S S S	-	-	-	-	-	
2	Brain-cord	-	-	-	-	5 6 7 7	6 6 7 S	S S S S	$10^{-6.3}$
	Spleen		6 6 6 D	6 6 7 D	S S S S	-	-	-	$10^{-3.5}$

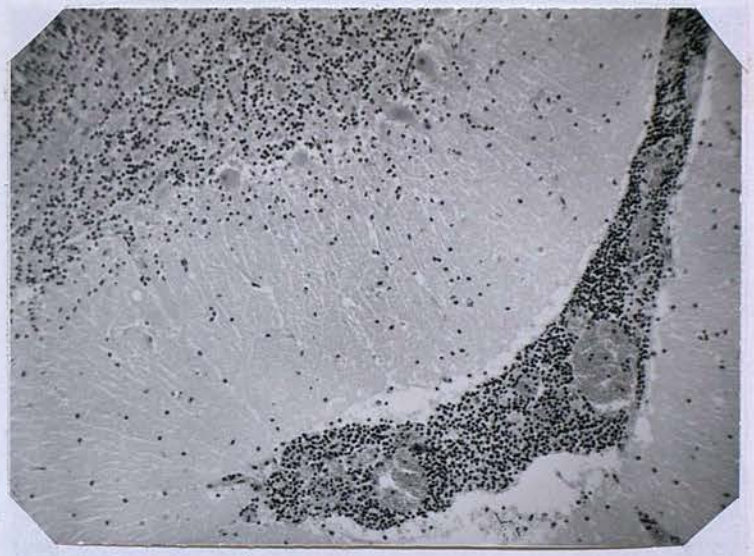
Figures = destruction of the mouse when showing typical louping-ill symptoms in days after inoculation.
D = death from undetermined cause.
S = survived for period of 15 days.
* = Log LD_{50} dilution /0.05 ml. inoculum.
- = not done.

(ii) Method of hyperimmunisation

Six sheep vaccinated against the disease were given repeated subcutaneous inoculations of living antigen. The antigen was a 10 per cent. tissue suspension of either brain pool in physiological saline (0.85 per cent. NaCl) and each sheep received a total of 620 ml. in 18 inoculations over a period of approximately 14 weeks. From 20 to 50 ml. of antigen was inoculated on 3 consecutive days at intervals of 2 to 3 weeks. The inoculum was freshly prepared prior to administration and was centrifuged at a R.C.F. of approximately 800 for 20 minutes, to eliminate coarse tissue particles. No local effect was observed at the sites of inoculation, but hyperimmunisation was abruptly discontinued at the fourteenth week when a sheep (No.282) receiving inoculations of normal brain in a parallel immunisation programme died of an encephalitis from which no infectious agent could be recovered. Histological examination of the brain of this sheep (figure 1) revealed severe lymphocytic infiltrations around blood vessels and some diffuse infiltration into the brain substance. No apparent neuron degeneration was present. It was concluded that the condition was an allergic encephalitis representing a neuro-paralytic accident.

Figure 1

The histopathology of allergic encephalitis in the central nervous system of sheep No. 282 following repeated subcutaneous inoculations with normal sheep brain. No infectious agent was found in these tissues of the inoculated sheep.



(a) Cerebellum. Severe lymphocytic infiltration around the blood vessels in the interfolial space. No apparent neuron degeneration is present. H. & E., x 110.

(b) Medulla. Diffuse infiltration of lymphocytic cells in the brain substance and around capillaries. H. & E., x 110.

(iii) Results of hyperimmunisation

The antibody responses achieved in these sheep one week following the course of inoculations described are shown in table 2. Antibody level was measured by the technique of Schwentker and Rivers (1933). The sera to be tested were mixed with equal volumes of falling tenfold dilutions, commencing from 1 in 5, of mouse brain virus in saline-serum. Serum-virus mixtures were incubated in vitro at room temperature for 2 hours followed by $\frac{1}{2}$ to 1 hour at 4°C. prior to inoculation intracranially, in 0.05 ml. volumes, into 4 anaesthetised mice per dilution; occasionally 2 mice per dilution were used. In each test of unknown sera a known normal serum was included as a control. Mice were kept under observation for a period of 15 days and titres were calculated and expressed as before. All samples of sera were filtered through G.S. Seitz pads and stored at -28°C. without preservatives.

From table 2 it will be seen that the best sera, those of sheep Nos. 265 and 266, protected mice from 10,000 or more mouse LD_{50} of virus as determined by the corresponding titration of normal serum. This in fact represents the amount of virus neutralised by 0.025 ml. of each serum.

Table 2

Mouse titrations of louping-ill neutralising antibody in sera of sheep hyperimmunised with homologous brain virus. The inoculum per mouse was 0.05 ml. comprising equal volumes of serum and virus.

Test	Sheep number	Virus - log dilution									Titre*	Number of LD_{50} neutralised by 0.025 ml. serum
		10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}	10^{-8}	10^{-9}	10^{-10}		
1	265	7 7 7 S	7 8 11 D	10 S SS	S S S S	-	-	-	-	-	$10^{-3.5}$	10,000
	266	6 6 7 8	7 7 9 S	S S S S	9 S S S	S S S S	-	-	-	-	$10^{-3.4}$	12,590
	267	7 7 7 10	7 8 10 S	7 10 S S	S S S S	-	-	-	-	-	$10^{-3.7}$	6,310
	Control	-	-	-	-	6 6 6 8	6 7 8 S	7 S S S	8 S S S	S S S S	$10^{-7.5}$	-
2	268	6 6 7 7	8 8 9 S	8 S S S	S S S S	-	-	-	-	-	10-3.5	3,162
	269	6 7 7 7	7 7 8 S	7 S S S	S S S S	-	-	-	-	-	$10^{-3.5}$	3,162
	270	7 8 9 9	8 14 S S	11 S S S	S S S S	-	-	-	-	-	$10^{-3.2}$	6,310
	Control	-	-	-	-	6 6 7 9	8 9 S S	S S S S	-	-	$10^{-7.0}$	-

Abbreviations as in table 1.

B. Convalescent sera

(i) Experimentally induced convalescent sera

The studies of Pool et al.(1930) and Greig et al.(1931) showed that louping-ill infection could be artificially induced in sheep by the inoculation of virus subcutaneously and by other routes. Subcutaneous infection was generally followed by an elevation in temperature, often diphasic, without other symptoms; in many instances, the febrile reaction coincided with the occurrence of viraemia. In some cases, however, symptoms indicative of involvement of the central nervous system appeared. The course of the disease following subcutaneous infection was unpredictable and ended either in death of the animal or its complete clinical recovery accompanied by the development of immunity to subsequent intracerebral inoculation of the agent.

In the present studies, a number of sheep were infected by the subcutaneous route. Several of these were vaccinated animals which nevertheless had developed the disease after subcutaneous challenge with the virus but 10 were fully susceptible sheep (as determined by the serum neutralisation test) of the Cheviot breed intentionally infected by the inoculation of 10^4 to 10^6 mouse LD_{50} of virus subcutaneously (table 3). Among these, sheep No.1316 contracted the disease so badly that it had to be destroyed; the remainder survived and were immune. Susceptibility and immunity were assessed in the light of the recommendations of The Neurotropic Virus Disease Commission of 1942 (Smadel, 1952), which regarded a neutralisation/

Table 3

Details of ten susceptible sheep and their infection by subcutaneous route with louping-ill virus.

Sheep		Serum titration in mice						Virus inoculum in mouse LD_{50}/ subcutaneously	Outcome of infection
		Virus - log dilution							
Number	Age in months	10^{-6}	10^{-7}	10^{-8}	10^{-9}	Titre *	Neutralisation index		
1315	32	66	78	77	-	$10^{-8.5}$		10^4	Survived
1316	"	67	8S	9S	-	$10^{-7.4}$	3.9	"	** L.I. - sacrificed
1318	"	67	67	SS	-	$10^{-7.5}$	3.1	"	Survived
1321	33	66	77	DS	-	$10^{-7.5}$	3.1	5×10^4	"
1324	"	67	77	SS	-	$10^{-7.5}$	3.1	"	"
Control		66	88	8S		$10^{-8.0}$			
1938	9	67	7S	7S	-	$10^{-7.4}$	3.9	"	"
1970	"	67	79	8 15	-	$10^{-8.5}$		"	"
Control		66	77	11 S		$10^{-8.0}$			
1150	24	7777	7889	SSSS	-	$10^{-7.5}$	5.0	10^5	"
Control		7777	78910	812SS	12SSS	$10^{-8.2}$			
1317	"	6667	679S	8SSS	-	$10^{-7.5}$		10^6	"
Control		567D	788S	SSSS		$10^{-7.3}$			
1323	"	668D	677S	99SS		$10^{-7.7}$	1.9	"	"
Control		6667	788D	610SS	SSSS	$10^{-8.0}$			

Abbreviations as in table 1.
** Louping ill.

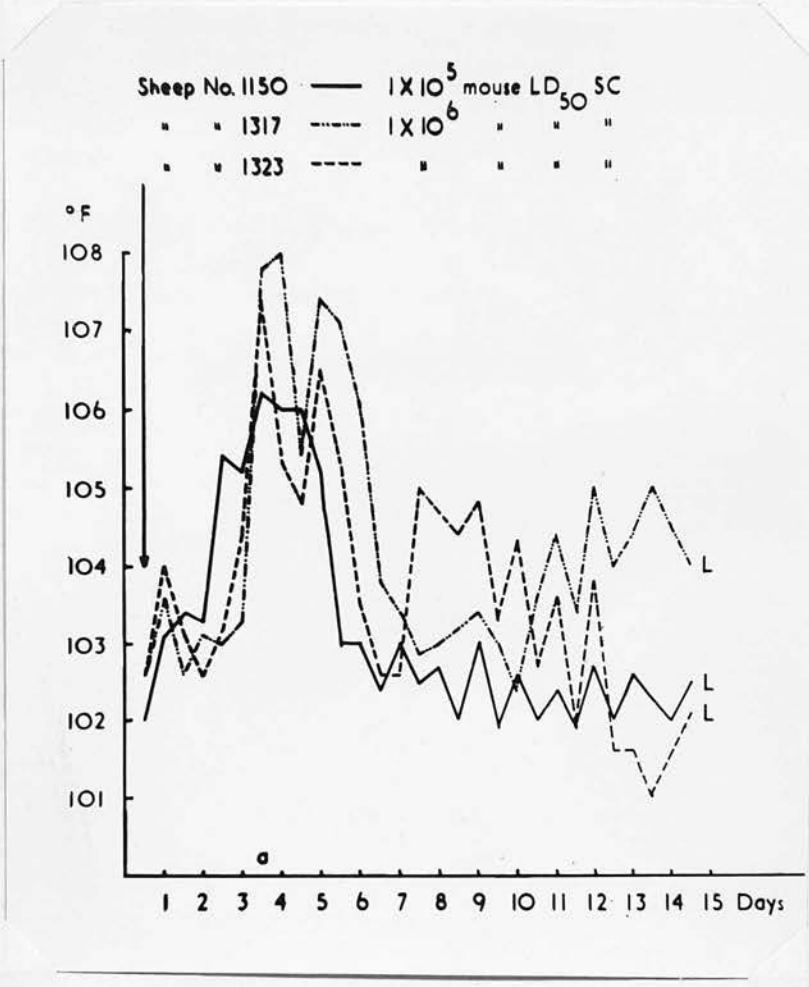
a neutralisation index of 1 to 9 as representing a negative serum, 10 to 49 equivocal, and 50 or more a positive serum. The titres recorded in table 3 established the susceptibility of the animals. The temperature reactions which occurred in these animals are illustrated in figures 2, 3 and 4. The classical diphasic temperature reaction associated with the disease is evidenced by sheep Nos. 1317 and 1323, figure 2, and No. 1324, figure 4. Viraemias were demonstrated in several of the animals. The need for recording temperatures twice daily is apparent in the charts of sheep No. 1315, figure 3, and No. 1321, figure 4, where abrupt significant rise and fall of temperatures occurred between morning or between evening readings on successive days.

That the central nervous system of the recovered sheep is immune to intracranial inoculation of virus was confirmed in the case of sheep Nos. 1938 and 1970, figure 4. Both of these animals survived intracerebral challenge with 10,000 mouse LD_{50} of virus administered 24 days after the original subcutaneous infection. Intracranial inoculation of the identical amount of virus in 2 susceptible sheep, Nos. 1930 and 2022, figure 5, proved fatal to both animals. They were sacrificed in the terminal stages of the disease on the sixth and seventh days, respectively, after infection. A pool of the central nervous systems and cervical cords of these sheep titred at $10^{-7.0}$ in mice.

As previously stated, sheep No. 1316 succumbed to subcutaneous infection and was sacrificed in the last stages of the disease. Brain tissue recovered from this animal, however/

Figure 2

Temperature graphs of sheep Nos. 1150, 1317, and 1323 following subcutaneous inoculation of virus of louping-ill.



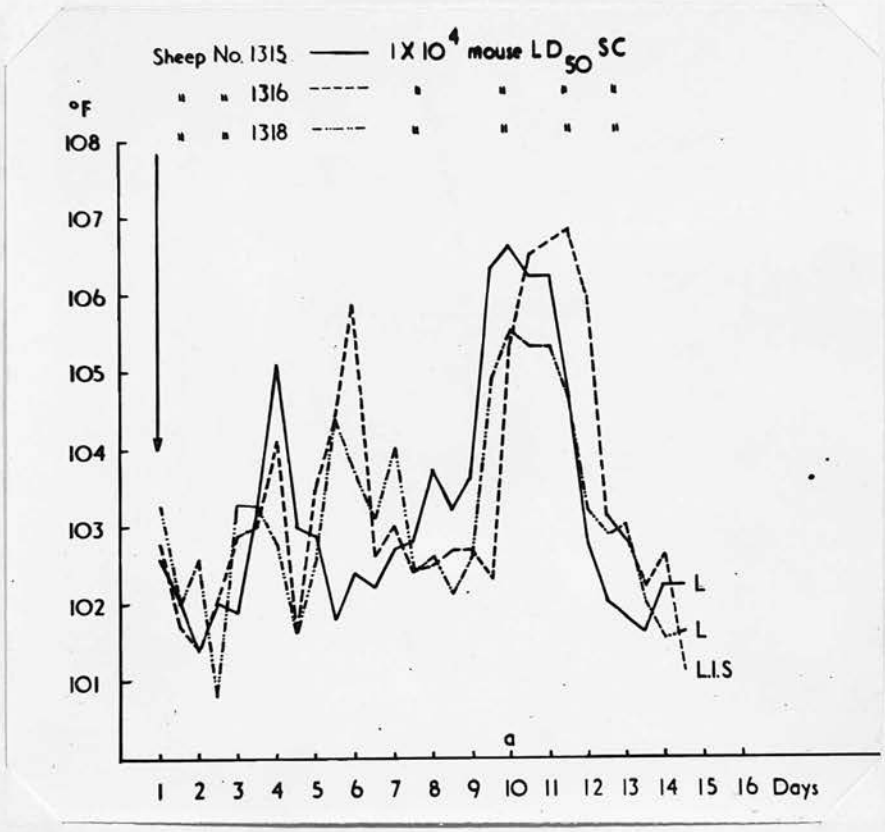
SC - Subcutaneously.

L - Lived.

a - Virus absent from the blood of sheep No. 1150
" present in " " " " " 1317
and 1323.

Figure 3

Temperature graphs of sheep Nos. 1315, 1316 and 1318 following subcutaneous inoculation of virus of louping-ill.

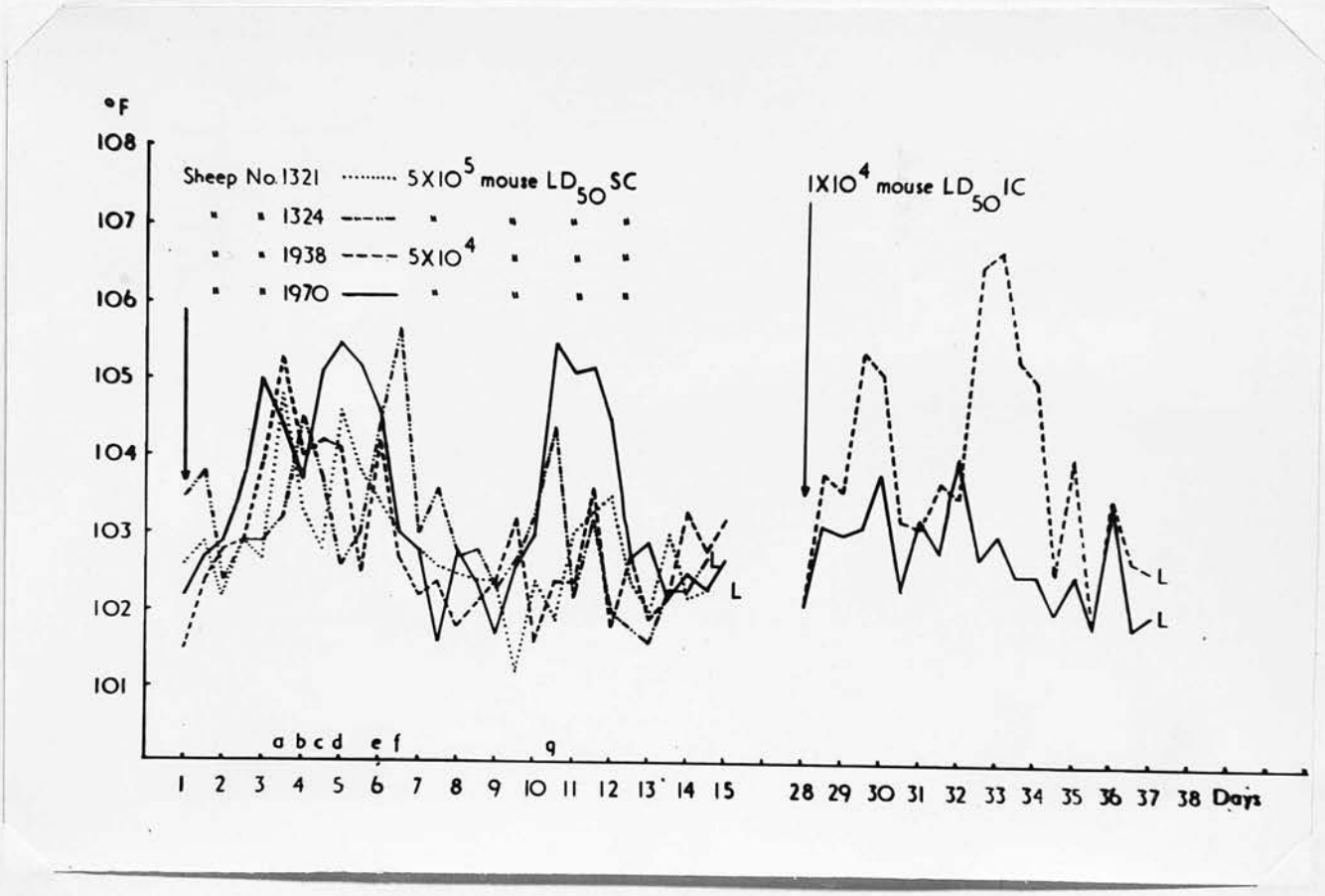


Abbreviations as in Figure 2.

L.I.S. - Louping-ill. Sacrificed.
a - Virus absent from the blood of all the sheep.

Figure 4

Temperature graphs of sheep Nos. 1321, 1324, 1938, and 1970 following subcutaneous inoculation of louping-ill and of the last 2 after subsequent intracranial challenge.



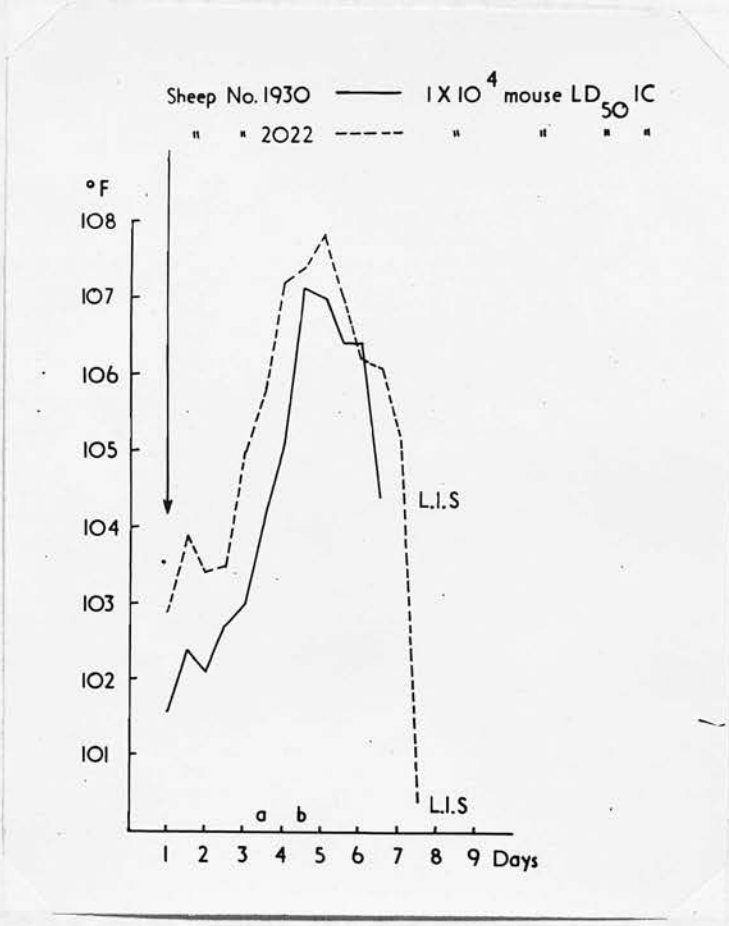
Abbreviations as in Figure 2.

IC - Intracranially.

a - Virus present in the blood of sheep No. 1321.
b - " " " " " " " " 1324, 1938, and 1970.
c - " absent from " " " " " 1970.
d - " " " " " " " " 1321.
" present in " " " " " 1970.
e - " " " " " " " " 1324.
" absent from " " " " " 1938.
f - " present in " " " " " 1324.
g - " absent from " " " " " 1970.

Figure 5

Temperature graphs of sheep Nos. 1930 and 2022 following intracranial inoculation of virus of louping-ill.



I.C. - Intracranially.
L.I.S.- Louping-ill. Sacrificed.
a - Virus present in the blood of sheep No. 2022.
b - " " " " " " sheep Nos. 1930 and 2022.

however, proved to be negative for virus when inoculated into mice in a 1 in 20 dilution. Histological examination of the brain of this animal (figure 6) showed severe perivascular cuffing and focal infiltrations of lymphocytes. In only a few isolated areas did the neurons exhibit degenerative changes such as pyknosis and chromatolysis. A single necrotic neuron was observed. By comparison, there was usually widespread destruction of Purkinje cells and chromatolysis and pyknosis of neurons in more typical louping-ill (figure 7).

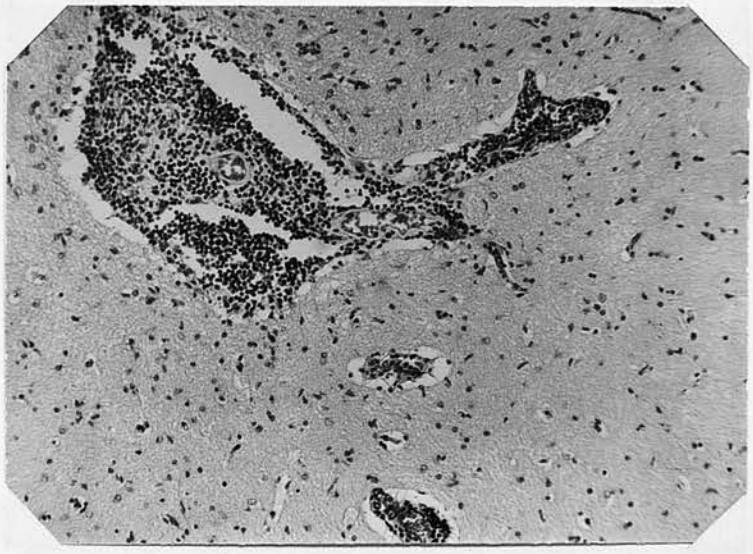
Table 4 shows the immune titres obtained in the group of susceptible sheep which were vaccinated and all of which took the disease when challenged subcutaneously with virus. Immunity was confirmed by a second challenge of virus intracranially. Although these titres had no doubt been augmented by the second challenge and, perhaps, to some degree as well by the antigen of the killed vaccine, these sheep were, nevertheless, regarded as convalescent for the purpose of these studies.

(ii) Natural convalescent sera

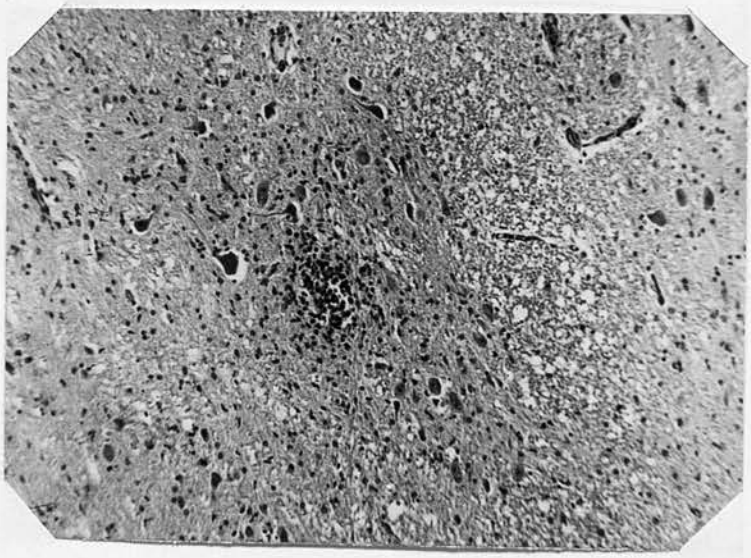
A number of sheep sera submitted from the field to the Moredun Institute for louping-ill diagnosis (by neutralisation test in the mouse) were available for testing for complement-fixing antibody.

Figure 6

Histopathology in the central nervous system of sheep No. 1316 destroyed *in extremis* exhibiting severe clinical symptoms of encephalitis following subcutaneous inoculation of louping-ill virus. Intracranial inoculations into mice yielded no evidence of virus in these tissues.

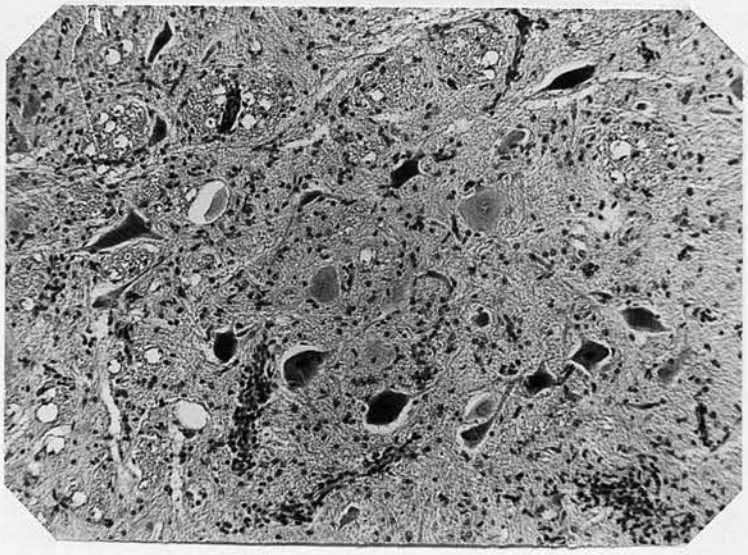


(a) Hippocampus. Severe perivascular infiltration. H. & E., x 110.



(b) Cerebral cortex. Focal infiltration of lymphocytes in association with neuron degeneration. H& E., 110.

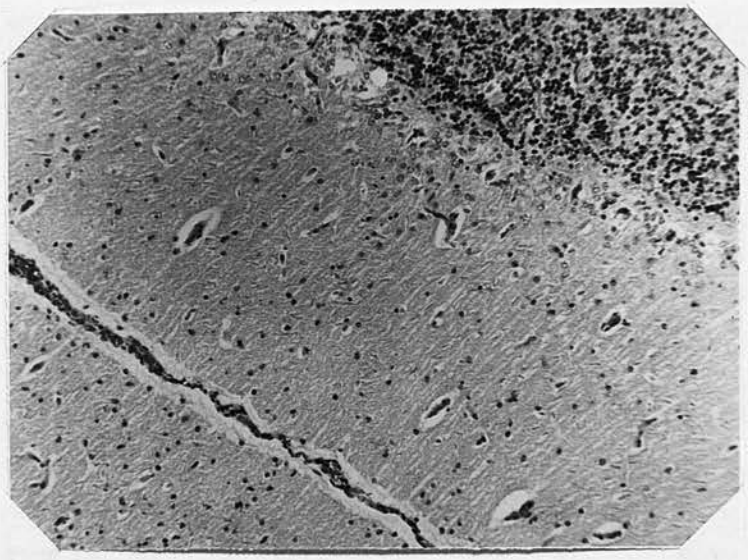
Figure 6 contd.



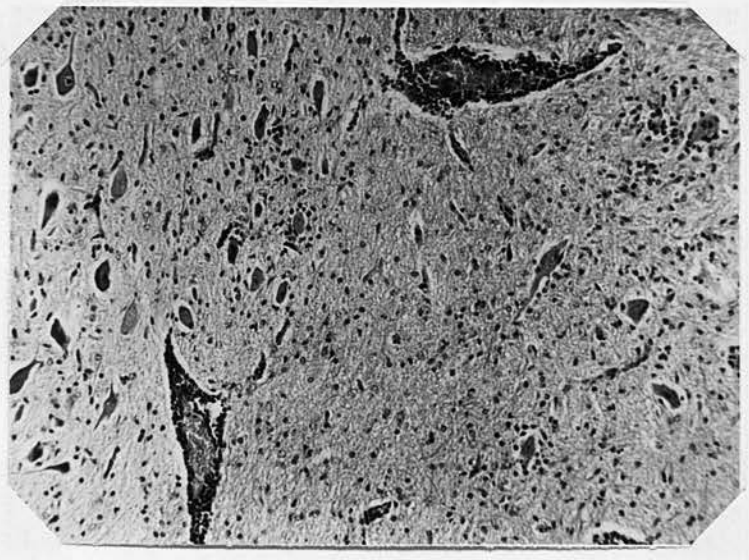
(c) Medulla. Focal infiltrations of lymphocytes. Pyknosis and chromatolysis of neurons and necrosis in a single instance. H.&E., x110.

Figure 7

The histopathology of louping-ill in the central nervous system of the sheep **(natural infection).**

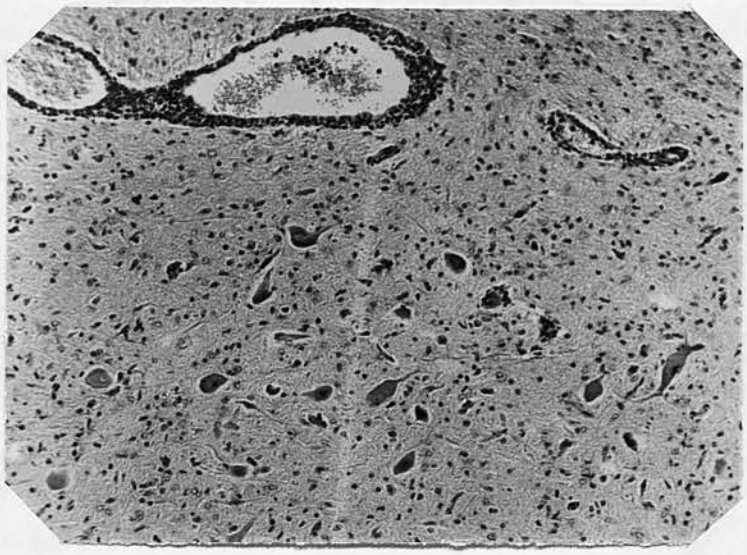


(a) Cerebellum. Slight perivascular infiltration around the blood vessels in the interfolial space. Sclerosis and complete disappearance of some of the Purkinje cells. H. & E., x 110.



(b) Medulla. Perivascular cuffing and an increase in glial cells around neurons. Widespread chromatolysis or pyknosis of the neurons. H. & E., x 110.

Figure 7 contd.



(c) Medulla. Perivascular cuffing and an increase in glial cells around neurons. Widespread chromatolysis or pyknosis of the neurons. H. & E., x 110.

Table 4

Mouse titrations of louping-ill neutralising antibody in the sera of sheep experimentally infected by the subcutaneous route after vaccination.

Sheep number	Virus - log dilution							Titre*	N.I.■
	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}	10^{-8}	10^{-9}		
2360	6 7 7 7	7 7 10 S	S S S S	S S S S	-	-	-	$10^{-4.3}$	7943
2361	6 6 6 6	7 8 8 9	D S S S	S S S S	-	-	-	$10^{-4.5}$	5012
2362	6 6 7 7	8 9 S S	S S S S	S S S S	-	-	-	$10^{-4.0}$	15850
2363	6 6 6 6	6 7 7 8	D 9 9 S	S S S S	-	-	-	$10^{-5.2}$	1000
2364	6 6 6 6	6 6 7 7	6 7 9 S	13 S S S	-	-	-	$10^{-5.5}$	501
2365	6 6 7 7	7 7 8 10	S S S S	S S S S	-	-	-	$10^{-4.5}$	5012
2366	6 6 6 7	6 6 7 9	7 7 7 S	S S S S	-	-	-	$10^{-5.3}$	794
Control	-	-	-	6 6 6 6	7 7 8 8	8 10 D S	S S S S	$10^{-8.2}$	

Abbreviations as in table 1.

■ Neutralisation index.

II. Virus

The virus of louping-ill employed throughout these studies was a strain originally isolated from sheep at the Moredun Institute several years previously. Since then, the agent had been propagated *in vivo* through sheep, mice and the developing chick embryo as well as *in vitro* in tissue culture of minced chick embryo suspended in Tyrode's solution.

A. Mouse Brain Virus

Virus was recovered from mice sacrificed at the height of the disease following intracerebral inoculation of the agent. Brains were harvested aseptically following etherisation and exsanguination of the sick mice and the tissues were stored in blended pools of about 30 gms. each at -28°C. The titre of virus in pools was $10^{-7.5}$ to $10^{-7.7}$ (table 5).

B. Tissue Culture Virus

In the present studies, the cultivation of virus in tissue culture was undertaken with a view to preparing a complement-fixing antigen; the method of culture employed was largely that of Wilson (1944).

Table 5

Results of multiple titrations in mice of 3 louping-ill mouse brain pools.

Pool	Test	Titre*	Mean titre*
No. 2	1	$10^{-7.5}$	
	2	$10^{-7.0}$	
	3	$10^{-7.3}$	
	4	$10^{-8.0}$	
	5	$10^{-8.0}$	$10^{-7.5}$
No. 3	1	$10^{-7.5}$	
	2	$10^{-8.0}$	
	3	$10^{-7.5}$	
	4	$10^{-7.3}$	
	5	$10^{-7.4}$	
	6	$10^{-8.0}$	
	7	$10^{-8.0}$	
	8	$10^{-8.0}$	$10^{-7.7}$
No. 4	1	$10^{-8.0}$	
	2	$10^{-7.8}$	
	3	$10^{-7.6}$	
	4	$10^{-8.0}$	
	5	$10^{-7.6}$	
	6	$10^{-8.2}$	
	7	$10^{-7.0}$	$10^{-7.7}$

* Log LD_{50} dilution /0.05 ml. inoculum.

(1) Materials and methods for growing virus in tissue culture.

(a) Virus

The strain was that which has already been described. Virus used to initiate these cultures was a 10^{-1} dilution of infected mouse brain (titre of $10^{-8.0}$ in mice), filtered through a gradocol membrane of 720 millimicrons A.P.D.

(b) Technique of suspended cell culture

1. Glassware. Pyrex glassware was employed except for pipettes. Glassware received no special treatment and was prepared through a hot soap solution followed by 2 washes in tap water. Sterilisation was carried out either in a dry air oven at 160°C. for 1 hour or by autoclaving at 20 lb. pressure for 20 minutes.

2. Preparation of media. Two physiological salt solutions and a synthetic mixture were used as nutrient media. Antibiotics were incorporated and on occasions sera were added.

Tyrode's solution. This solution was prepared as follows:-

NaCl, 8 gm.; KCl, 0.2 gm.; $CaCl_2$, 0.2 gm.; $MgCl_2$, 0.1 gm.; NaH_2PO_4, 0.05 gm.; $NaHCO_3$, 1.0 gm.; glucose, 1.0 gm.; distilled water, 1000 ml. Salts of analytical reagent grade were employed (Analar). The solution was stored unsterilised in convenient quantities with the glucose fraction excluded. Just prior to use, the glucose was added and the whole sterilised by filtration through a Seitz pad.

Hanks' balanced salt solution. Hanks' balanced salt solution was prepared as described by Weller et al., (1952). Two stock solutions were prepared as follows:-

(a) NaCl, 160 gm., KCl, 8 gm., Mg $SO_4.7H_2O$, 2 gm., $MgCl_2.6H_2O$, 2 gm.; (or Mg $SO_4.7H_2O$, 4 gm. in place of the two latter salts); distilled water 800 ml. This solution was combined with one of $CaCl_2$, 2.8 gm.; distilled water, 100 ml. The volume of the whole was made up to 1000 ml. with distilled water, 2 ml. chloroform were added, and the solution stored at 4°C.

(b) $Na_2HPO_4.12H_2O$, 3.04 gm.; KH_2PO_4, 1.2 gm.; dextrose, 20 gm.; distilled water 800 ml. One hundred millilitres of a 0.4 per cent. solution of phenol red were added and the volume of the whole made up to 1000 ml. Chloroform was added as before and the solution stored at 4°C. The phenol red solution was prepared by slowly adding N/20 NaOH to 1 gm. of powdered phenol red until the solution was complete and deep red in colour. The volume was made up to 250 ml. with distilled water.

Hanks' balanced salt solution was compounded by adding 1 volume each of the stock solutions (a) and (b) to 18 volumes of distilled water. The whole was sterilised at 9 lb. pressure for 10 minutes and 0.5 ml. of a sterile 1.4 per cent. $NaHCO_3$ solution added to each 20 ml. of Hanks' balanced salt solution before use. The solution of $NaHCO_3$ was sterilised at 9 lb. pressure for 10 minutes and stored at 4°C.

Synthetic Mixture No. 199. This medium (Morgan, J.F., et al., 1950) was purchased from Glaxo Laboratories Ltd.

Sera. Sheep serum was prepared from blood drawn from an animal free from neutralising antibody to louping-ill and was sterilised by filtration through a Seitz G.S. pad. Normal horse serum (without preservative) was purchased from Evans Medical Supplies Ltd. All sera were stored frozen at -28°C.

Antibiotics. Glaxo 'Crystamycin' composed of penicillin, streptomycin, and dihydrostreptomycin was used. It was added to media prepared with Tyrode's and Hanks' balanced salt solutions to give a final concentration per ml. of 100 units of penicillin and 0.05 milligram each of streptomycin and dihydrostreptomycin. Synthetic Mixture No. 199 already contained antibiotics when purchased.

3. General procedure. All manipulations were performed on the open bench. Contamination was rarely encountered.

4. Tissues. Chick embryonic tissues were obtained from eggs incubated for 9 to 11 days at 38° to 39°C. The eggs were totally immersed in alcohol for approximately 5 minutes before being opened at the air space. Embryos and chorio-allantoic membranes were removed aseptically, washed several times in medium, and minced with a pair of scissors until the fragments were reduced to 2 to 4 mm. in diameter.

5. Preparation of cultures. Cultures were prepared both in Roux (12 x 20 cm.) and 50 ml. Erlenmeyer flasks. Roux flasks were seeded with 0.5 ml. minced tissue suspended in 50 ml. of nutrient medium while 50 ml. flasks received 4 to 5 drops of tissue (from a capillary pipette 2 mm. internal diameter) which was suspended in 6 ml. of medium. Flasks were stoppered with cotton plugs or red rubber bungs which fitted tightly. Virus was inoculated at the time of preparation of the culture and flasks were incubated at 37°C., Roux flasks on their sides. A minimum of 3 flasks of any culture system were prepared at any one time, 2 for virus inoculation while the third served as an uninoculated control. The pH of cultures in flasks stoppered with cotton plugs was unadjusted after preparation. The pH of filtered Tyrode's solution was approximately 8.4 and fell to 8.2 in newly prepared cultures. It was 8.0 to 8.2 after 4 days of incubation with or without virus. Synthetic Mixture No. 199 was pH 6.8 or less when received and increased in alkalinity to pH 7.8 or more on incubation in culture. Freshly prepared cultures of Hanks' balanced salt solution were pH 7.6 but rapidly became more alkaline on incubation. The pH of uninoculated control cultures followed the same course of changes encountered in virus infected ones. In cultures tightly stoppered with rubber bungs Synthetic Mixture No.199 was adjusted to pH 7.4 to 7.6 as the tendency was for all media to become less alkaline on incubation.

6. Passage of virus. Virus was propagated serially in cultures through several passages. Passage of the virus was effected by transferring pooled undiluted infected medium (harvested on the fourth to fifth day of incubation) from one group of cultures to each of a group of newly prepared ones. Inocula for Roux and 50 ml. flask cultures were 2.0 ml. and 0.2 ml. respectively.

7. Sterility test. Material from each flask was plated on to blood agar 24 hours before being harvested, that is, on the third or fourth day of incubation. Contamination was rarely encountered.

(ii) Experimental results of growing virus in tissue culture

(a) Multiplication of louping-ill virus during serial passage.

The first 6 passages of virus were grown in chick embryo in Tyrode's solution seeded in Roux flasks (part 1 of table 6). By the sixth passage, the original virus inoculum (titre of $10^{-8.0}$) was diluted 2.4×10^{9} but the yield of virus titred at $10^{-5.0}$; the cultures were clearly supporting virus multiplication. Virus growth in cultures containing Hanks' balanced salt solution was identical to that in cultures containing Tyrode's solution. The addition of normal sheep serum to the extent of 10 per cent. in nutrient medium (Tyrode's solution) did not alter the yield of virus.

Part 2 of table 6 presents the virus titres recovered from passages 7 to 13 and 1 to 4 in tissues of chick embryo and chorio-allantois respectively, suspended in various nutrient/

Table 6

Details of passages of louping-ill virus in tissue cultures of chick embryo and chick chorio-allantois suspended in various nutrient media. The initial viral inoculum was a $10^{-1.0}$ dilution of infected mouse brain of titre $10^{-8.0}$. The tissues used at each horizontal plane were obtained from the same eggs.

Part 1

Passage	Cumulative dilution of original virus inoculum	Culture system less physiological salt solution	Virus yield - mouse titre* Physiological salt solution	
			Tyrode's	Hanks'
1	2.5×10^{2}	Whole embryo + 10 per cent. sheep serum	-	-
2	6.2×10^{3}	" "	$10^{-6.0}$ or >	$10^{-6.0}$ or >
3	1.5×10^{5}	" "	$10^{-5.5}$	$10^{-5.5}$
4	3.9×10^{6}	" "	$10^{-6.0}$	$10^{-6.0}$
5 A	9.7×10^{7}	" + 10 per cent. heated (60°C/30 min.) sheep serum	$10^{-4.3}$	-
B		Whole embryo only	$10^{-4.5}$	-
6	2.4×10^{9}	Decapitated embryo only	$10^{-5.0}$	-

Part 2

Passage	Cumulative dilution of original virus inoculum	Virus yield - mouse titre*					
		Decapitated chick embryo			Chick chorio-allantois		
		Tyrode's Solution	Mixture No. 199	Mixture No. 199 + serum▲	Tyrode's Solution	Mixture No. 199	Mixture No. 199 + serum▲
7	7.3×10^{10}	$10^{-4.5}$	$10^{-4.5}$	-	-	-	-
B					$10^{-3.4}$		
8	2.1×10^{12}	$10^{-3.0}$	-	-	1st passage	-	-
					$10^{-4.2}$		
9	6.5×10^{13}	$10^{-4.5}$	$10^{-5.0}$	-	2nd passage	-	-
					$10^{-4.0}$		
10	1.9×10^{15}	$10^{-5.5}$ or >	$10^{-5.5}$ or >	-	3rd passage	-	-
					$10^{-5.5}$		
11	5.9×10^{16}	$10^{-4.0}$	$10^{-4.0}$	$10^{-5.0}$	4th passage	$10^{-4.5}$	$10^{-5.0}$
12	1.7×10^{18}	$10^{-5.2}$	-	-	-	-	-
13	5.3×10^{19}	$10^{-4.5}$	-	-	-	-	-

* Log LD_{50} dilution /0.05 ml. inoculum.

- Not done.

▲ 2 per cent. heated (56°C/30 min.) horse serum.

nutrient media. The tissues used at each horizontal plane in the table under reference were obtained from the same eggs. The virus used for initiating infection in cultures of chorio-allantoic membrane was seventh passage culture virus propagated in chick embryo in Tyrode's solution. By the fourth passage in cultures of chorio-allantois, the initial inoculum of virus (titre of $10^{-4.5}$) was diluted 7.1×10^5 but the titre of virus yielded was $10^{-5.5}$. This was regarded as proof of the multiplication of the agent in this culture system.

The mean titre of virus yielded by 13 passages in cultures of chick embryo in Tyrode's solution was $10^{-4.8}$ while those of comparable cultures of chick embryo in Tyrode's solution and in Synthetic Mixture No. 199 (passages 7, 9, 10 and 11) were $10^{-4.6}$ and $10^{-4.7}$, respectively. The mean titres of virus grown in cultures of chick embryo (passages 8 to 11) and chorio-allantois (passages 1 to 4), both suspended in Tyrode's solution, were identical, namely, $10^{-4.2}$; these culture systems were comparable except for the number of passages. The mean titre of all cultures was $10^{-4.7}$.

(b) The viability of cultures in regard to virus multiplication.

The yield of virus in cultures infected when grown as opposed to those infected when freshly prepared was compared. Whereas cultures infected at the time of preparation grew virus to the titre of $10^{-4.4}$ after 4 days of incubation, the agent could not be demonstrated in a $10^{-3.0}$ dilution (the lowest/

lowest dilution tested) of pooled fluids harvested after similar incubation from comparable cultures grown for 4 days prior to the introduction of virus.

(c) Viability of louping-ill culture virus

No special study was undertaken to determine the stability of the virus in culture; however, re-examination of certain cultures, stored under refrigeration, revealed an appreciable fall in titre. Virus stored at -28°C. survived for approximately 18 weeks in media free of serum. In one instance, however, virus in culture medium containing 2 per cent. horse serum survived for 30 weeks.

(d) Identification of the virus grown in tissue culture.

The identification of the agent propagated in tissue cultures was established by neutralisation with louping-ill hyperimmune sheep serum. The serum dilution technique was used in which tenfold dilutions of sera were mixed with an equal volume of virus so that the serum-virus inocula contained approximately 300 mouse LD_{50} of culture virus. Serum-virus mixtures were incubated in vitro for 2 hours at room temperature followed by $\frac{1}{2}$ to 1 hour at 4°C. prior to inoculation into mice. The marked neutralisation of the agent (eleventh passage) by louping-ill hyperimmune serum was regarded as proof of its identity (table 7).

111. Diluent

Physiological saline, 0.85 per cent. NaCl in distilled water, served as the diluent for the reagents used in the complement-fixation test.

Table 7

Neutralisation in mice of louping ill virus grown for 11 passages in tissue culture of chick embryo in Tyrode's solution, by specific hyperimmune sheep serum.

Mouse LD_{50} of culture virus	Sheep serum						
		Log dilution					
	Type	Undil	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}
300	Normal	$4/4$ *	$4/4$	$4/4$	$4/4$	$4/4$	$4/4$
	Louping-ill hyperimmune	$0/4$	$0/4$	$1/4$	$3/4$	$4/4$	$4/4$

* Numerator - number of mice killed showing typical symptoms of louping ill up to the fifteenth day after inoculation of serum-virus mixtures.

Denominator - number of mice inoculated with serum-virus mixtures.

IV. Complement

The complement employed in these studies was the preserved guinea-pig serum marketed by Messrs. Burroughs Wellcome. In preparing complement for use in the test it was diluted with 7 volumes of distilled water to give the equivalent of a 1 in 10 dilution of fresh complement in saline. From this, a series of sub-dilutions was prepared in diluent. Complement was titrated at the commencement of each test in the presence of diluent alone as well as in the presence of each antigen.

V. Amboceptor

The amboceptor used was the glycerinated haemolytic serum for sheep corpuscles also obtained from Messrs. Burroughs Wellcome. The average titre of this serum was 1 in 1500; prior to use it was diluted to give a suitable excess of 5 M.H.D. per test dose.

VI. Erythrocytes

Sheep blood cells drawn from the jugular vein were used. After defibrination, the erythrocytes were obtained by three successive centrifugal washings in diluent. Cells were used up to the fifth day after being drawn and received 2 further washings whenever stored overnight. The routine method for preparing sensitised cell suspensions was to take 2.0 ml. washed and packed erythrocytes and to suspend them in 50 ml. of diluent. To this suspension was added 50 ml. of diluent containing 0.3 ml. amboceptor. This mixture was held at room temperature for 15 minutes before inclusion in the test.

VII. Preparation of antigens

A. Mouse brain antigens

(i) Freeze-thawed antigen

A number of antigens was prepared from infected mouse brain after the method described by Casals and Palacios (1941); only minor modifications were introduced in order to suit the equipment available. Brain tissue was weighed and emulsified in 5 times its weight by volume of a diluent consisting of 0.85 per cent. NaCl in distilled water plus 2 per cent. normal inactivated (56°C. for 30 minutes) guinea-pig serum. Emulsification was accomplished in a Griffith tube as well as in an 'atomix' for 3 minutes. The emulsion was allowed to stand at 4°C. for 20 hours when it was centrifuged horizontally at a R.C.F. of approximately 1400 for 30 minutes.

The supernatant liquid was removed to plastic tubes in which it was alternately frozen and thawed 8 times in a dry-ice alcohol mixture and 37°C. bath, respectively. A precipitate formed which was removed by centrifugation at a R.C.F. of approximately 5750 in an angle head centrifuge for 1 hour at 4°C. Merthiolate was added to give a final concentration of 1 in 10,000 parts of antigen. Identical control antigens were prepared from normal mouse brain (normal antigen). Antigens were stored at -28°C.

(ii) Freeze-dried extracted antigen

Louping-ill infected and normal mouse brain tissues were dried from the frozen state by two methods. In the first method a 20 per cent. brain emulsion in double distilled/

distilled water was shell-frozen in McCartney bottles in 6.5 ml. amounts after the emulsion was allowed to stand overnight at 4°C. Five such amounts were placed in a glass dessicator containing calcium chloride and the jar rapidly evacuated with a Cenco Hyvac pump. The tissue dried in about 18 hours. In the second method, brain was dried in a model 3 P.S./A. vacuum drier marketed by W. Edwards & Co. Several 25 gm. amounts of blended undiluted brain, pre-frozen in Petri dishes, were dried simultaneously. The vacuum created was less than 0.2 mm. of mercury and during the first 2 hours of drying the wall of the drying chamber was heated to 37° to 40°C. The vacuum was maintained for a total of 24 hours. Dried brain, which was reduced to approximately one fifth of its wet weight, was stored tightly stoppered at -28°C. There was no adverse effect on the titre of virus dessicated in the commercial drier, the more lengthy process involving also the application of heat to the drying chamber.

The preparation of the antigen was completed by extracting the dried brain at room temperature with a weight by volume of organic solvent 9 times that of the equivalent weight of wet brain. The organic solvents used were ether, acetone, and benzol. Three consecutive extractions for half an hour each were made using manual agitation and the tissue was recovered by light centrifugation. Residual solvent was rapidly dried in vacuo. The dry extracted tissue was resuspended to give a dilution of 1 part of the equivalent wet brain in 9 parts of saline (0.85 per cent. NaCl.).

Following/

Following rehydration for 2 hours at room temperature or overnight at 4°C. the material was centrifuged at a R.C.F. of approximately 5750 for 1 hour in an angle head centrifuge in the cold. The supernatant liquid was the final antigen.

B. Tissue culture antigen

Undiluted infected culture fluid cleared by horizontal centrifugation at a R.C.F. of approximately 1400 for 30 minutes was used as antigen. Antigens were prepared as soon as the results of titrations in mice were known and the oldest culture virus employed as a complement-fixing antigen was 36 days after the suspension of incubation. Normal control culture antigens were prepared from fluids taken from uninfected cultures (normal antigen).

VIII. Procedure for the complement-fixation test

All of the constituents in the complement-fixation test were used in 0.2 ml. volumes. Twofold dilutions of each serum under test were prepared and complement (2M.H.D.) and antigen added to each tube in that order; the tubes were incubated at 4°C. for 18 hours. At the completion of the cold incubation the tubes were allowed to stand at room temperature for 30 minutes and then the haemolytic system was added. The total volume in each tube was 0.8 ml. The test was read after further incubation at 37°C. for 1 hour in a bath. Complete haemolysis was expressed as 0, the absence of haemolysis (complete fixation) as 4, and intermediate degrees of fixation as ½, 1, 2, and 3. The titre of a serum was expressed as the dilution showing 2 or more fixation.

In addition to the titrations of complement in the presence of each antigen and in the presence of diluent only, each serum was tested (in its lowest dilution) in the presence of 2 M.H.D. of complement for anti-complementary action. A known positive and a known negative louping-ill serum (as established by the neutralisation test) were included in each test. Finally, each serum was tested with a louping-ill antigen as well as an uninfected normal antigen.

EXPERIMENTAL RESULTS

1. Anti-complementary action of antigens

At the commencement of each test complement was titrated in the presence of each louping-ill and normal antigen as well as in the presence of diluent only. All ingredients in these titrations were used in 0.2 ml. amounts and titrations were prepared in duplicate. One complete set was incubated at 37°C. for 1 hour, the haemolytic system added, and the test read after further incubation as before. The second set was incubated along with the test proper at 4°C. for 18 hours. The amount of complement representing 2 M.H.D. was determined from the first set of titrations.

A. Mouse brain antigens

Mouse brain antigens prepared by the two methods described were usually not anti-complementary at the end of 18 hours incubation in the cold. Table 8 shows typical titrations of 2 pairs of freeze-thawed louping-ill and normal mouse brain antigens following short incubation (1 hour at 37°C.) and/

Table 8

The results of titrations of complement in the presence of 2 pairs of freeze-thawed louping-ill and normal mouse brain antigens after incubation at 37°C. for 1 hour and at 4 °C. for 18 hours.

Antigen	Millilitres of complement							
	Incubation 37°C., 1 hour				Incubation 4°C., 18 hours			
	0.0033	0.0028	0.0023	0.0020	0.0033	0.0028	0.0023	0.0020
Louping-ill No. 1	0	0	±	2	0	0	0	1
Normal No. 1	0	0	±	2	0	0	0	1
Louping-ill No. 2	0	0	±	2	0	0	0	1
Normal No. 2	0	0	±	1	0	0	0	±
None	0	0	±	1	0	0	0	1

0 = complete haemolysis

4 = absence of haemolysis (Complete fixation)

± 1, 2, and 3 = intermediate degrees of fixation.

and overnight incubation in the cold (18 hours at 4°C.). It will be seen that a slight improvement occurred in the titre of complement incubated overnight. In neither set of titrations were the antigens anti-complementary.

B. Tissue culture antigen

Some difficulty was experienced in rendering tissue culture antigens free from anti-complementary effects (table 9). Antigen prepared from fourth passage virus (see part 1 of table 6) grown in whole chick embryo tissue suspended in Tyrode's solution containing 10 per cent. sheep serum proved to be anti-complementary following both short and overnight incubation. Such antigen, centrifuged at a R.C.F. of approximately 36,190 for 30 minutes at 4°C. in an angle head, or heated to 56°C. for 30 minutes, continued to be strongly anti-complementary. Similarly, antigen prepared from fifth passage virus, grown in whole embryo in Tyrode's solution incorporating 10 per cent. heated sheep serum (60°C. for 30 minutes), and from fifth passage virus, grown in whole embryo in Tyrode's solution, were both anti-complementary after overnight incubation; uninfected culture fluids (normal antigens) from identical culture systems were equally anti-complementary (titrations not shown). Sixth passage virus, grown in decapitated chick embryo in Tyrode's solution, however, gave rise to antigens which proved to be completely free from anti-complementary effects; uninfected fluid from comparable cultures was the same.

Table 9

The results of complement titrations in the presence of a number of tissue culture antigens after incubation at 37°C. for 1 hour and at 4°C. for 18 hours.

Test	Antigen			Millilitres of complement														
	Type	Passage	Treatment	Incubation - 37°C., 1 hour								Incubation - 4°C., 18 hours						
				0.01	0.0080	0.0060	0.0041	0.0033	0.0028	0.0023	0.0020	0.01	0.0080	0.0060	0.0041	0.0033	0.0028	0.0023	0.0020
1	L.I.■ - culture	Fourth ✱	None	-	-	-	2	3	4	4	-	2	4	4	4	4	4	4	-
			Centrifuged - R.C.F. 36, 190/30 minutes	-	-	-	2	3	4	4	-	1	3	4	4	4	4	4	-
	None			-	-	-	0	1	3	4	-	-	-	-	0	±	2	3	-
2	L.I. - culture	Fourth ✱	Heated to 56°C./30 minutes	0	0	0	1	2	3	4	-	±	3	4	4	4	4	4	-
	None			0	0	0	0	0	±	3	-	0	0	0	0	±	1	4	-
3	L.I. - culture	Fifth-A ✱	None	-	-	0	0	0	1	2	-	-	-	0	±	1	2	3	-
		Fifth-B ✱	None	-	-	0	0	0	0	1	-	-	-	0	0	±	1	2	-
	None			-	-	0	0	0	0	1		-	-	0	0	0	0	±	-
4	L.I. - culture	Sixth ✱	None	-	-	-	-	0	0	±	1	-	-	-	-	0	0	1	3
	Uninoculated - culture		None	-	-	-	-	0	0	±	1	-	-	-	-	0	0	1	3
	L.I. - freeze-thawed		None	-	-	-	-	0	0	1	2	-	-	-	-	0	0	1	3
	None			-	-	-	-	0	±	1	2	-	-	-	-	0	±	2	3

Key as in table 8.

■ Louping ill

✱ See part 1 of table 6

\- Not done

11. Potency of antigens

A. Mouse brain antigens

The potency of mouse brain antigens was determined by 'checkerboard' titration with serum from a sheep hyperimmunised with homologous brain antigen; two-fold dilutions of serum were tested with twofold dilutions of the antigen. Two M.H.D. of complement, as determined by titration in the presence of the lowest dilution of antigen employed, were used. The results of simultaneous 'checkerboard' titrations of 2 freeze-thawed antigens (Nos. 22 and 24) with the same hyperimmune serum,No. 265d,are shown in table 10. In both instances, the optimum dilution of the antigens was 1 in 4 or a 5 per cent. emulsion. Subsequent specific tests, however, in which antigen was used in this strength,led to an unduly large number of sera exhibiting the 'zoning phenomenon'. Retitration of antigen No. 24 with a second hyperimmune serum,No. 269d,(table 11) revealed its optimum dilution to be 1 in 2 or a 10 per cent. emulsion. Table 12 shows an identical experience with a freeze-dried benzol-extracted mouse brain antigen when titrated simultaneously with the 2 above hyperimmune sheep sera; whereas the optimum dilution with serum No. 265d was 1 in 4, it was 1 in 2 with serum No. 269d. Ideally, each antigen should be titrated in 'checkerboard' fashion with each serum under test; however, this is impractical. It would seem advisable, nevertheless, that titrations should be done with at least 2 sera. Eventually, all louping-ill mouse brain antigens were routinely prepared as 10 per cent. emulsion.

Table 10

'Checkerboard' titrations of 2 freeze-thawed louping-ill mouse brain antigens with a hyperimmune sheep serum

Antigen			Serum dilution (No. 265d)				
No.	Dilution		1:4	1:8	1:16	1:32	1:64
22	Undil.	20 per cent.	4	4	4	2	0
	1:2	10 per cent.	4	4	4	4	0
	1:4	5 per cent.	4	4	4	4	0
	1:8	2.5 per cent.	3	3	2	0	0
24	Undil.	20 per cent.	4	4	4	±	0
	1.2	10 per cent.	4	4	4	4	0
	1:4	5 per cent.	4	4	4	4	0
	1:8	2.5 per cent.	3	3	3	±	0
	None		0	-	-	-	-

Key as in table 8.

- Not done.

Table 11

'Checkerboard' titration of a freeze-thawed louping-ill mouse brain antigen with a hyperimmune sheep serum

Antigen			Serum dilution (No. 269d)				
No.	Dilution		1:4	1:8	1:16	1:32	1:64
24	Undil.	20 per cent.	4	4	4	±	0
	1:2	10 per cent.	4	4	4	2	0
	1:4	5 per cent.	±	4	4	3	0
	1:8	2.5 per cent.	0	±	1	1	±
	None		0	0	-	-	-

Key as in table 8.

- Not done.

Table 12

'Checkerboard' titration of a freeze-dried benzol-extracted louping-ill mouse brain antigen with 2 hyperimmune sheep sera.

Antigen dilution		Serum dilution													
		Serum No. 265d							Serum No. 269d						
		1:4	1:8	1:16	1:32	1:64	1:128	1:256	1:4	1:8	1:16	1:32	1:64	1:128	1:256
Undil.	20 per cent.	4	4	4	4	4	3	0	4	4	4	4	4	0	0
1:2	10 per cent.	4	4	4	4	4	3	0	4	4	4	4	4	0	0
1:4	5 per cent.	4	4	4	4	4	3	0	2	4	4	4	4	0	0
1:8	2.5 per cent.	3	4	4	3	3	1	0	0	! =	1	2	2	0	0
None		0	0	-	-	-	-	-	0	0	-	-	-	-	-

Key as in table 8.

- Not done.

B. Tissue culture antigen

Specific tests were carried out with tissue culture antigens prepared both from fifth and sixth passage cultures which titrated at $10^{-4.3}$ and $10^{-5.0}$, respectively, in mice. They were tested undiluted and both failed to fix complement. Table 13 shows the results of the test with sixth passage antigen as well as fixation obtained between the same hyper-immune sheep serum and a freeze-thawed louping-ill mouse brain antigen. Although fixation took place at 1 in 64 with the latter antigen, none occurred with the tissue culture antigen. Attempts to use louping-ill culture virus as a complement-fixing antigen were discontinued in view of the absence of fixation with an antigen which was of higher titre ($10^{-5.0}$) than the mean yield of virus grown in all cultures ($10^{-4.7}$).

III. Specificity of the reaction

A. Normal and immune sera

Sheep sera were heated to 60°C. for 30 minutes and to 65° and 68°C. for 20 minutes each, to determine the effects on non-specific fixation when they were tested with freeze-thawed louping-ill and normal mouse brain antigens (table 14). Normal serum, heated to 60°C., reacted non-specifically at 1 in 8 and 1 in 4 with the louping-ill and normal antigens, respect-

ively/

Table 13

Results of complement-fixation tests with sixth passage tissue-culture and freeze-thawed mouse brain antigens, and sheep sera.

Antigen	Serum dilution							
	Hyperimmune serum							Normal Serum
	1:2	1:4	1:8	1:16	1:32	1:64	1:128	1:2
Sixth passage louping-ill culture	1	0	0	0	0	0	0	2
Normal culture	0	-	-	-	-	-	-	0
Freeze-thawed louping-ill mouse brain	4	4	4	4	4	2	0	0
Freeze thawed normal mouse brain	0	-	-	-	-	-	-	0
None	0	-	-	-	-	-	-	0

Key as in table 8.

- Not done.

Table 14

The effects on specific and non-specific fixation of heating sheep sera to various temperatures; these are tested with freeze-thawed louping-ill and normalmouse brain antigens.

Antigen	Serum									
	Heat Treatment		Dilution							
			Hyperimmune					Normal		
	Temp. °C.	Time min.	1:4	1:8	1:16	1:32	1:64	1:4	1:8	1:16
Louping-ill	60	30	4	4	4	4	0	4	3	0
Normal			±	0	0	-	-	4	1	0
None			0	-	-	-	-	0	-	-
Louping-ill	65	20	4	4	4	4	0	1	0	0
Normal			0	0	0	-	-	±	0	0
None			0	-	-	-	-	0	-	-
Louping-ill	68	20	4	4	4	±	0	0	0	0
Normal			0	0	0	-	-	0	0	0
None			0	-	-	-	-	0	-	-

Key as in table 8.

- Not done.

respectively, while specific fixation with louping-ill hyperimmune serum,heated to the same temperature,was 1 in 32 with louping-ill antigen. Neither serum was anticomplementary in its lowest dilution tested (1 in 4). Heating to 65°C. eliminated almost completely the non-specific fixation with normal serum and both antigens without affecting the specific fixation with hyperimmune serum and louping-ill antigen. Heating to 68°C. rendered normal serum completely free from non-specific fixation. However, specific fixation with hyperimmune serum and louping-ill antigen was reduced from 1 in 32 to 1 in 16. Unless otherwise stated, the heating of sera to 65°C. for 20 minutes was routinely practised.

Table 15 shows an instance of a sheep serum which reacted non-specifically with both louping-ill and normal freeze-dried benzol-extracted antigens and a subsequent sample, from the same animal,which did not show any trace of non-specific fixation when tested with the same antigens. Both samples were free from anticomplementary action in their lowest dilutions tested (1 in 2) and were treated identically.

B. Mouse brain antigens

(i) Freeze-thawed antigen

Certain sera reacted non-specifically with freeze-thawed louping-ill and normal mouse brain antigen even after being heated to 65°C. for 20 minutes. Ultra-centrifugation of the antigen was attempted in order to improve specificity. Table 16 presents the results of simultaneous tests with antigen centrifuged/

Table 15.

Variation in non-specific fixation exhibited by two samples of serum drawn at different times from the same normal sheep (free from louping-ill neutralising antibody) when tested with freeze-dried benzol-extracted louping-ill and normal mouse brain antigens.

Antigen	Serum dilution													
	Hyperimmune - drawn 3/4/56						Normal							
							Drawn 30/5/56				Drawn 9/8/56			
	1:2	1:4	1:8	1:16	1:32	1:64	1:2	1:4	1:8	1:16	1:2	1:4	1:8	1:16
Louping-ill	4	4	4	4	4	0	4	4	±	0	0	0	0	0
Normal	0	0	0	0	0	-	4	4	0	0	0	0	0	0
None	0	-	-	-	-	-	0	-	-	-	0	-	-	-

Key as in table 8.

- Not done.

Table 16

The effects of single and multiple ultracentrifugation of freeze-thawed louping-ill and normal mouse brain antigens on specific and non-specific fixation when tested with sheep sera.

Antigen		Serum dilution							
		Hyperimmune					Normal		
Centrifugation - R.C.F./30 minutes	Type	1:4	1:8	1:16	1:32	1:64	1:4	1:8	1:16
1 x 36,190	Louping-ill	4	4	4	4	0	2	±	0
	Normal	±	0	0	-	-	1	0	0
3 x 36,190	Louping-ill	4	4	4	0	0	2	0	0
	Normal	±	0	0	-	-	1	0	0
	None	0	-	-	-	-	0	-	-

Key as in table 8.

- Not done.

centrifuged once at a R.C.F. of approximately 36,190 for 30 minutes in an angle head centrifuge in the cold and that centrifuged in an identical manner on 3 consecutive occasions; in the latter preparation the supernatant was removed to fresh centrifuge tubes after each centrifugation. Multiple ultracentrifugation had no diminishing effect on non-specific fixation but the titre of specific fixation was appreciably reduced, namely, from 1 in 32 to 1 in 16. Similar results were obtained when the rate of a single ultracentrifugation of antigen was increased beyond a R.C.F. of approximately 56,550 for 30 minutes (table 17). Ultracentrifugation at a R.C.F. of approximately 81,430 or at 144,700 for 30 minutes reduced specific fixation from 1 in 32 to 1 in 8 with little alteration in the degree of non-specific fixation. Boiling the antigen for 20 minutes resulted in the complete destruction of antigenicity. No further attempt was made to improve freeze-thawed brain antigen and its use was abandoned except for comparative purposes with freeze-dried extracted antigens.

(ii) Freeze-dried extracted antigen

Freeze-dried brain antigen was extracted with 3 organic solvents, namely, ether, acetone, and benzol. The action of the solvents was to remove the lipids which are generally accepted as being responsible, in part at least, for the non-specific action of brain tissue. Table 18 presents the effects on non-specific fixation when freeze-dried/

Table 17

The effects of different rates of ultracentrifugation of freeze-thawed louping-ill and normal mouse brain antigens on specific and non-specific fixation when tested with sheepsera.

Antigen		Serum dilution							
		Hyperimmune					Normal		
Centrifugation - R.C.F./30 minutes	Type	1:4	1:8	1:16	1:32	1:64	1:4	1:8	1:16
36,190	Louping-ill	4	4	4	4	0	2	1	0
	Normal	1	±	0	-	-	1	±	0
56,550	Louping-ill	4	4	4	3	0	1	±	0
	Normal	±	±	0	-	-	1	±	0
81,430	Louping-ill	3	3	±	0	0	1	±	0
	Normal	±	0	0	-	-	±	±	0
144,700	Louping-ill	2	2	±	0	0	1	±	0
	Normal	±	0	0	-	-	1	±	0
	None	0	-	-	-	-	0	-	-

Key as in table 8
- Not done.

Table 18

The effects on non-specificity of extracting louping-ill and normal freeze-dried mouse brain antigens with ether, acetone,and benzol. Simultaneous tests with louping-ill and normal freeze-thawed mouse brain antigens are shown.

Antigen			Serum dilution															
			Hyperimmune							Normal No. 1				Normal No. 2				
Preparation	Solvent	Type	1:2	1:4	1:8	1:16	1:32	1:64	1:128	1:2	1:4	1:8	1:16	1:2	1:4	1:8	1:16	
Freeze-dried	Ether	L.I.*	4	4	4	4	4	3	0	0	0	0	0	-	-	-	-	
		N.■	±	0	0	0	-	-	-	±	±	0	0	-	-	-	-	
	Acetone	L.I.	4	4	4	4	4	4	0	3	3	±	0	-	-	-	-	
		N.	0	0	0	0	-	-	-	0	0	0	0	-	-	-	-	
	Benzol	L.I.	4	4	4	4	4	4	±	0	0	0	0	-	-	-	-	
		N.	0	0	0	0	-	-	-	0	0	0	0	-	-	-	-	
Freeze-thawed		L.I.	4	4	4	4	4	±	0	4	3	0	0	0	0	0	0	
		N.	±	±	0	0	-	-	-	2	1	0	0	0	0	0	0	
None			0	-	-	-	-	-	-	0	-	-	-	0	-	-	-	

Key as in table 8.

* Louping-ill

■ Normal

- Not done.

freeze-dried louping-ill and normal mouse brain antigens, extracted with these solvents, were tested with sheep sera. Simultaneous tests with freeze-thawed louping-ill and normal mouse brain antigens are also shown. Normal serum No. 1 reacted non-specifically at 1 in 4 and 1 in 2 with freeze-thawed louping-ill and normal antigens, respectively, and the titre of specific fixation with hyperimmune serum and louping-ill antigen was 1 in 32. Amongst the extracted antigens, louping-ill extracted with acetone reacted non-specifically with normal serum No. 1 at 1 in 4 while those extracted with ether and benzol proved to be completely free from non-specific fixation. The titre of specific fixation with hyperimmune serum and all extracted louping-ill antigens was 1 in 64. Further investigations were carried out on benzol-extracted antigen.

It soon became evident, however, that freeze-dried antigens extracted manually with benzol were not always free from non-specific fixation when tested with various sheep sera. This is shown in table 19 where normal serum No. 1913 reacted non-specifically with freeze-thawed antigens but not so with benzol-extracted freeze-dried antigens whereas normal serum No. 1314 reacted non-specifically with both the freeze-thawed and the freeze-dried antigens. Increasing the time of extraction or the quantity of solvent did not further reduce the occurrence of non-specific fixation.

It was found, however, that substitution of rapid mechanical agitation (with a magnetic stirrer) for that done by hand, during extraction with solvent, resulted in antigens which/

Table 19

Complement-fixation tests with freeze-thawed and freeze-dried benzol-extracted louping-ill and normal mouse brain antigens and sheep sera, illustrating the partial improvement of specificity with the latter antigens.

Antigen		Serum dilution															
Preparation	Type	Hyperimmune								Normal No. 1913				Normal No. 1314			
		1:2	1:4	1:8	1:16	1:32	1:64	1:128	1:256	1:2	1:4	1:8	1:16	1:2	1:4	1:8	1:16
Freeze-thawed	Louping-ill	4	4	4	4	4	4	±	0	4	4	3	±	4	4	3	0
	Normal	0	0	0	0	-	-	-	-	3	3	1	0	4	3	1	0
Freeze-dried benzol-extracted	Louping-ill	4	4	4	4	4	4	4	±	0	0	0	-	4	4	4	-
	Normal	0	0	0	-	-	-	-	-	0	0	0	-	4	4	2	-
None		0	-	-	-	-	-	-	-	0	-	-	-	0	-	-	-

Key as in table 8.

- Not done.

which did not react non-specifically with sheep sera. However, the foregoing work with antigens manually agitated during extraction was completed and recorded before the possibilities of mechanical agitation were investigated. The superiority of antigens mechanically agitated during extraction is shown in table 20. Whereas normal serum No. 1314 did not react non-specifically with mechanically agitated antigens, non-specific fixation occurred at 1 in 4 and 1 in 2 with louping-ill and normal antigens, respectively, agitated manually during extraction. Unless otherwise stated, the remainder of these studies was carried out with antigens extracted with benzol by mechanical agitation.

IV. Relationship of serum complement-fixing and neutralising antibodies.

A number of tests was carried out to show the relationship between serum complement-fixing and serum neutralising antibodies. Since all of these tests were carried out simultaneously with louping-ill and normal antigens, they also served to indicate the incidence of non-specific fixation which could be expected amongst sheep sera in general. The following group classification of sera was based on the result of the neutralisation test in mice, a neutralisation index of 1 to 9 representing a negative serum, 10 to 49 equivocal, and 50 or more a positive serum.

A. Hyperimmune sera

Complement-fixation tests were carried out with 2 lots of sera drawn from 5 of the 6 sheep hyperimmunised with homologous brain virus (table 21). Complement-fixing antibody was present in high titre (1 in 64 to 1 in 128) in all the d samples which were secured 1 week after the termination of/

Table 20

The effects on non-specific fixation of extracting, with benzol, freeze-dried louping-ill and normal mouse brain antigens by manual and mechanical agitation.

Antigen		Serum dilution																	
		Hyperimmune								Normal No. 1314					Normal No. 1001				
Agitation during extraction	Type	1:2	1:4	1:8	1:16	1:32	1:64	1:128	1:256	1:2	1:4	1:8	1:16	1:32	1:2	1:4	1:8	1:16	1:32
Manual	Louping-ill	4	4	4	4	4	3	0	0	4	4	1	0	0	0	0	0	0	0
	Normal	0	0	0	-	-	-	-	-	3	1	0	0	0	0	0	0	0	0
Mechanical	Louping-ill	4	4	4	4	4	3	0	0	0	0	0	0	0	0	0	0	0	0
	Normal	±	0	0	-	-	-	-	-	0	0	0	0	0	0	0	0	0	0
None		0	-	-	-	-	-	-	-	0	-	-	-	-	0	-	-	-	-

Key as in table 8.

- Not done.

Table 21

Complement-fixation tests with freeze-dried benzol-extracted louping-ill and normal mouse brain antigens and hyperimmune sheep sera. The d samples were drawn one week after the termination of hyperimmunisation and e samples 15 weeks later. Neutralisation indices of the former sera are shown.

Antigen	Serum									
	Number	Dilution								Neutralisation index
		1:2	1:4	1:8	1:16	1:32	1:64	1:128	1:256	
L.I.*	265 d	4	4	4	4	4	4	4	±	10,000
N.■		0	0	0	-	-	-	-	-	
L.I.	" e	4	4	4	2	0	0	0	0	-
N.		0	0	0	-	-	-	-	-	
L.I.	267 d	4	4	4	4	4	4	4	1	16,310
N.		0	0	0	-	-	-	-	-	
L.I.	" e	4	4	4	4	1	0	0	0	-
N.		0	0	0	-	-	-	-	-	
L.I.	268 d	4	4	4	4	4	4	±	0	3,162
N.		0	0	0	-	-	-	-	-	
L.I.	" e	0	0	0	0	0	0	0	0	-
N.		0	0	0	-	-	-	-	-	
L.I.	269 d	4	4	4	4	4	4	±	0	3,162
N.		0	0	0	-	-	-	-	-	
L.I.	" e	±	4	2	±	0	0	0	0	-
N.		0	0	0	-	-	-	-	-	
L.I.	270 d	4	4	4	4	4	4	4	±	6,310
N.		0	0	0	-	-	-	-	-	
L.I.	" e	1	4	4	4	±	0	0	0	-
N.		0	0	0	-	-	-	-	-	
L.I.	Normal	0	0	0	-	-	-	-	-	
N.	control	0	0	0	-	-	-	-	-	

Key as in table 8.
* Louping ill
■ Normal
- Not done.

of hyperimmunisation. The e samples, however, which were taken 15 weeks after the d, evidenced a marked fall in titres (0 to 1 in 16). It was concluded that a striking decline in circulating complement-fixing antibody was the consequence of withholding any further inoculation of antigen into hyper-immunised sheep. Non-specific fixation did not occur.

B. Convalescent sera

(i) Experimentally induced convalescent sera.

The convalescent sera of the 9 susceptible sheep which were experimentally infected with virus were examined in some detail to show, if possible, the appearance, persistence, and decline of both serum complement-fixing and neutralising antibodies (table 22).

Complement-fixing antibody was clearly demonstrated (2 or more fixation) in all 9 sheep. Disregarding sheep No. 1315, which was not tested between the third and tenth week of convalescence, complement-fixing antibody made its appearance 2 to 6 weeks after subcutaneous infection and the maximum titres recorded varied from 1 in 8 (sheep Nos. 1150, 1321, and 1970) to 1 in 32 (sheep No. 1938). Antibody persisted for less than a total of 6 weeks in 1 animal (sheep No. 1321) and for less than a total of 21 weeks in 3 others (sheep Nos. 1150, 1315, and 1318). In another animal (sheep No. 1317) it persisted for less than a total of 47 weeks while in the remainder, (sheep Nos. 1323, 1324, 1938, and 1970) it was demonstrated to the end of the periods of observation which varied from 28 to 47 weeks.

Table 22

Complement-fixation with the sera of nine sheep* experimentally infected with louping-ill virus and freeze-dried benzol-extracted louping ill and normal mouse brain antigens. Some neutralisation indices are shown but in none of the tests do they bear direct comparison with those of the control sera.

Test No. 1, Sheep No. 1150

Antigen	Serum										
	Number	When drawn - weeks following infection	Dilution								Neutralisation index
			1:2	1:4	1:8	1:16	1:32	1:64	1:128	1:256	
L.I.**	1150	0	0	0	0	0	0	0	0	-	
N.**			0	0	0	-	-	-	-	-	
L.I.	"	2	0	0	0	0	0	0	0	-	19
N.			0	0	0	-	-	-	-	-	
L.I.	"	3	3	3	2	±	0	0	0	-	63
N.			0	0	0	-	-	-	-	-	
L.I.	"	4	3	3	2	±	0	0	0	-	199
N.			0	0	0	-	-	-	-	-	
L.I.	"	5	3	3	2	±	0	0	0	-	158
N.			0	0	0	-	-	-	-	-	
L.I.	"	6	3	3	2	±	0	0	0	-	199
N.			0	0	0	-	-	-	-	-	
L.I.	"	7	3	3	2	±	0	0	0	-	-
N.			0	0	0	-	-	-	-	-	
L.I.	"	11	2	1	0	0	0	0	0	-	-
N.			0	0	0	-	-	-	-	-	
L.I.	"	21	0	0	0	0	0	0	0	-	-
N.			0	0	0	-	-	-	-	-	
L.I.	Immune control		4	4	4	4	4	4	0	0	10,000
N.			0	0	0	-	-	-	-	-	
L.I.	Normal control		0	0	0	0	-	-	-	-	
N.			0	0	0	0	-	-	-	-	

Table 22 Contd.

Test No. 2, Sheep No. 1317

Antigen	Serum										
	Number	When drawn - weeks following infection	Dilution								Neutralisation index
			1:2	1:4	1:8	1:16	1:32	1:64	1:128	1:256	
L.I.***	1317	0	0	0	0	0	0	0	0	-	
N.*			0	0	0	-	-	-	-	-	
L.I.	"	1	0	0	0	0	0	0	0	-	63
N.			0	0	0	-	-	-	-	-	
L.I.	"	2	4	4	4	2	±	0	0	-	79
N.			0	0	0	-	-	-	-	-	
L.I.	"	3	4	4	4	3	0	0	0	-	316
N.			0	0	0	-	-	-	-	-	
L.I.	"	4	4	4	4	2	0	0	0	-	794
N.			0	0	0	-	-	-	-	-	
L.I.	"	5	4	4	4	2	0	0	0	-	-
N.			0	0	0	-	-	-	-	-	
L.I.	"	9	4	4	4	2	0	0	0	-	-
N.			0	0	0	-	-	-	-	-	
L.I.	"	19	4	3	±	0	0	0	0	-	-
N.			0	0	0	-	-	-	-	-	
L.I.	"	23	3	2	±	0	0	0	0	-	-
N.			0	0	0	-	-	-	-	-	
L.I.	"	28	3	2	±	0	0	0	0	-	-
N.			0	0	0	-	-	-	-	-	
L.I.	"	48	1	1	±	0	0	0	0	-	-
N.			0	0	0	-	-	-	-	-	
L.I.	Immune control		4	4	4	4	4	4	0	0	10,000
N.			0	0	0	-	-	-	-	-	
L.I.	Normal control		0	0	0	0	-	-	-	-	
N.			0	0	0	0	-	-	-	-	

Table 22 Contd.

Test No. 3, Sheep No. 1323

Antigen	Serum										
	Number	When drawn - weeks following infection	Dilution								Neutralisation index
			1:2	1:4	1:8	1:16	1:32	1:64	1:128	1:256	
L.I.**	1323	0	0	0	0	0	0	0	0	-	
N.*			0	0	0	-	-	-	-	-	
L.I.	"	1	0	0	0	0	0	0	0	-	125
N.			0	0	0	-	-	-	-	-	
L.I.	"	2	4	4	4	3	1	0	0	-	501
N.			0	0	0	-	-	-	-	-	
L.I.	"	3	4	4	4	3	1	0	0	-	158
N.			0	0	0	-	-	-	-	-	
L.I.	"	4	4	4	4	3	1	0	0	-	251
N.			0	0	0	-	-	-	-	-	
L.I.	"	8	4	4	4	3	! =	0	0	-	-
N.			0	0	0	-	-	-	-	-	
L.I.	"	18	4	4	4	1	0	0	0	-	-
N.			0	0	0	-	-	-	-	-	
L.I.	"	22	4	4	4	1	0	0	0	-	-
N.			0	0	0	-	-	-	-	-	
L.I.	"	29	4	4	4	1	0	0	0	-	-
N.			0	0	0	-	-	-	-	-	
L.I.	"	47	4	4	4	! =	0	0	0	-	-
N.			0	0	0	-	-	-	-	-	
L.I.	Immune control		4	4	4	4	4	4	0	0	10,000
N.			0	0	0	-	-	-	-	-	
L.I.	Normal control		0	0	0	0	-	-	-	-	
N.			0	0	0	0	-	-	-	-	

Table 22 Contd.

Test No. 4, Sheep No. 1315

Antigen	Serum									
	Number	When drawn - weeks following infection	Dilution							Neutralisation index
			1:2	1:4	1:8	1:16	1:32	1:64	1:128	1:256	
L.I.**	1315	0	0	0	0	0	0	0	0	-	
N.*			0	0	0	-	-	-	-	-	
L.I.	"	1	0	0	0	0	0	0	0	-	199
N.			0	0	0	-	-	-	-	-	
L.I.	"	2	0	0	0	0	0	0	0	-	50
N.			0	0	0	-	-	-	-	-	
L.I.	"	3	0	0	0	0	0	0	0	-	251
N.			0	0	0	-	-	-	-	-	
L.I.	"	10	2	1	±	0	0	0	0	-	316
N.			0	0	0	-	-	-	-	-	
L.I.	"	16	1	±	0	0	0	0	0	-	-
N.			0	0	0	-	-	-	-	-	
L.I.	"	20	±	±	0	0	0	0	0	-	-
N.			0	0	0	-	-	-	-	-	
L.I.	"	26	±	0	0	0	0	0	0	-	-
N.			0	0	0	-	-	-	-	-	
L.I.	"	34	±	±	0	0	0	0	0	-	1,000
N.			0	0	0	-	-	-	-	-	
L.I.	Immune control		4	4	4	4	4	4	1	0	10,000
N.			0	0	0	-	-	-	-	-	
L.I.	Normal control		0	0	0	0	-	-	-	-	
N.			0	0	0	0	-	-	-	-	

Table 22 Contd.

Test No. 5, Sheep No. 1318

Antigen	Serum									
	Number	When drawn - weeks following infection	Dilution							Neutralisation index
			1:2	1:4	1:8	1:16	1:32	1:64	1:128	1:256	
L.I.**	1318	0	0	1	±	0	0	0	0	-	1
N.*			0	0	0	-	-	-	-	-	
L.I.	"	1	±	±	0	0	0	0	0	-	100
N.			0	0	0	-	-	-	-	-	
L.I.	"	2	2	2	2	2	±	0	0	-	63
N.			0	0	0	-	-	-	-	-	
L.I.	"	3	2	3	3	2	±	0	0	-	100
N.			0	0	0	-	-	-	-	-	
L.I.	"	10	2	2	2	±	0	0	0	-	316
N.			0	0	0	-	-	-	-	-	
L.I.	"	16	±	±	±	0	0	0	0	-	-
N.			0	0	0	-	-	-	-	-	
L.I.	"	20	±	±	0	0	0	0	0	-	-
N.			0	0	0	-	-	-	-	-	
L.I.	"	26	1	±	±	0	0	0	0	-	-
N.			0	0	0	-	-	-	-	-	
L.I.	"	34	1	±	0	0	0	0	0	-	5,012
N.			0	0	0	-	-	-	-	-	
L.I.	Immune control		4	4	4	4	4	4	1	0	10,000
N.			0	0	0	-	-	-	-	-	
L.I.	Normal control		0	0	0	0	-	-	-	-	
N.			0	0	0	0	-	-	-	-	

Table 22 Contd.

Test No. 6, Sheep No. 1321

Antigen	Serum										
	Number	When drawn - weeks following infection	Dilution								Neutralisation index
			1:2	1:4	1:8	1:16	1:32	1:64	1:128	1:256	
L.I.**	1321	0	0	0	0	2	0	0	0	-	
N.*			0	0	0	-	-	-	-	-	
L.I.	"	1	0	0	0	0	0	0	0	-	15
N.			0	0	0	-	-	-	-	-	
L.I.	"	2	0	0	0	0	0	0	0	-	316
N.			0	0	0	-	-	-	-	-	
L.I.	"	3	1	1	1	±	0	0	0	-	794
N.			0	0	0	-	-	-	-	-	
L.I.	"	4	1	±	±	-	0	0	0	-	794
N.			0	0	0	-	-	-	-	-	
L.I.	"	5	3	3	2	0	0	0	0	-	1,000
N.			0	0	0	-	-	-	-	-	
L.I.	"	6	3	3	±	0	0	0	0	-	316
N.			0	0	0	-	-	-	-	-	
L.I.	"	10	±	±	0	0	0	0	0	-	-
N.			0	0	0	-	-	-	-	-	
L.I.	"	14	±	±	0	0	0	0	0	-	-
N.			0	0	0	-	-	-	-	-	
L.I.	"	20	±	0	0	0	0	0	0	-	-
N.			0	0	0	-	-	-	-	-	
L.I.	"	28	0	0	0	0	0	0	0	-	316
N.			0	0	0	-	-	-	-	-	
L.I.	Immune control		4	4	4	4	4	4	1	0	10,000
N.			0	0	0	-	-	-	-	-	
L.I.	Normal control		0	0	0	0	0	-	-	-	
N.			0	0	0	0	0	-	-	-	

Table 22 Contd.

Test No. 7, Sheep No. 1324

Antigen	Serum										
	Number	When drawn - weeks following infection	Dilution								Neutralisation index
			1:2	1:4	1:8	1:16	1:32	1:64	1:128	1:256	
L.I.**	1324	0	0	0	0	0	0	0	0	-	1
N.*			0	0	0	-	-	-	-	-	
L.I.	"	1	0	0	0	0	0	0	0	-	10
N.			0	0	0	-	-	-	-	-	
L.I.	"	2	0	4	1	±	0	0	0	-	6
N.			0	0	0	-	-	-	-	-	
L.I.	"	3	3	4	3	2	0	0	0	-	6
N.			0	0	0	-	-	-	-	-	
L.I.	"	4	3	3	3	1	0	0	0	-	158
N.			0	1	0	-	-	-	-	-	
L.I.	"	5	3	3	3	±	±	0	0	-	100
N.			0	0	0	-	-	-	-	-	
L.I.	"	6	4	4	4	2	0	0	0	-	100
N.			0	0	0	-	-	-	-	-	
L.I.	"	10	4	4	4	2	0	0	0	-	-
N.			0	3	0	-	-	-	-	-	
L.I.	"	14	4	4	4	4	±	0	0	-	-
N.			0	0	0	-	-	-	-	-	
L.I.	"	20	4	4	4	4	±	0	0	-	-
N.			1	0	0	-	-	-	-	-	
L.I.	"	28	4	4	4	3	±	0	0	-	1,000
N.			0	0	0	-	-	-	-	-	
L.I.	Immune control		4	4	4	4	4	4	1	0	10,000
N.			0	0	0	-	-	-	-	-	
L.I.	Normal control		0	0	0	0	0	-	-	-	1
N.			0	0	0	0	0	-	-	-	

Table 22 Contd.

Test No. 8, Sheep No. 1938

Antigen	Serum										
	Number	When drawn - weeks following infection	Dilution								Neutralisation Index
			1:2	1:4	1:8	1:16	1:32	1:64	1:128	1:256	
L.I.[XX]	1938	0	0	0	0	0	0	0	0	-	
N.[X]			0	0	0	-	-	-	-	-	
L.I.	"	1	0	0	0	0	0	0	0	-	79
N.			0	0	0	-	-	-	-	-	
L.I.	"	2	0	0	0	0	0	0	0	-	501
N.			0	0	0	-	-	-	-	-	
L.I.	"	3	0	0	0	0	0	0	0	-	125
N.			0	0	0	-	-	-	-	-	
L.I.	"	4	0	0	0	0	0	0	0	-	794
N.			0	0	0	-	-	-	-	-	
L.I.	"	5	1	1	±	0	0	0	0	-	6,310
N.			0	0	0	-	-	-	-	-	
L.I.	"	6	4	4	4	4	3	±	0	-	2,512
N.			0	0	0	-	-	-	-	-	
L.I.	"	10	4	4	4	4	3	±	0	-	-
N.			0	0	0	-	-	-	-	-	
L.I.	"	14	4	4	4	4	3	±	0	-	-
N.			0	0	0	-	-	-	-	-	
L.I.	"	20	4	4	4	4	2	0	0	-	-
N.			0	0	0	-	-	-	-	-	
L.I.	"	28	3	3	2	1	0	0	0	-	7,943
N.			0	0	0	-	-	-	-	-	
L.I.	Immune control		4	4	4	4	4	3	0	0	10,000
N.			0	0	0	-	-	-	-	-	
L.I.	Normal control		0	0	0	0	0				
N.			0	0	0	0	0				

Table 22 Contd.

Test No. 9, Sheep No. 1970

Antigen	Serum										
	Number	When drawn - weeks following infection	Dilution								Neutralisation index
			1:2	1:4	1:8	1:16	1:32	1:64	1:128	1:256	
L.I.**	1970	0	0	0	0	0	0	0	0	-	
N.*			0	0	0	-	-	-	-	-	
L.I.	"	1	0	0	0	0	0	0	0	-	
N.			0	0	0	-	-	-	-	-	
L.I.	"	2	1	1	1	1	0	0	0	-	39
N.			0	0	0	-	-	-	-	-	
L.I.	"	3	4	4	4	±	0	0	0	-	39
N.			0	0	0	-	-	-	-	-	
L.I.	"	4	4	4	3	±	0	0	0	-	251
N.			0	0	0	-	-	-	-	-	
L.I.	"	5	4	4	3	±	0	0	0	-	100
N.			0	0	0	-	-	-	-	-	
L.I.	"	6	4	4	4	±	0	0	0	-	100
N.			0	0	0	-	-	-	-	-	
L.I.	"	10	4	4	3	±	0	0	0	-	-
N.			0	0	0	-	-	-	-	-	
L.I.	"	14	4	4	3	±	0	0	0	-	-
N.			0	0	0	-	-	-	-	-	
L.I.	"	20	4	4	4	1	0	0	0	-	-
N.			4	3	±	-	-	-	-	-	
None			4	-	-	-	-	-	-	-	
L.I.	"	28	4	4	2	0	0	0	0	-	199
N.			3	1	0	-	-	-	-	-	
None			3	-	-	-	-	-	-	-	
L.I.	Immune control		4	4	4	4	4	4	±	0	10,000
N.			0	0	0	-	-	-	-	-	
L.I.	Normal control		0	0	0	0	-	-	-	-	
N.			0	0	0	0	-	-	-	-	

Key as in table 8.

Neutralising antibody indicative of infection was present in the sera of 5 animals (sheep Nos. 1315, 1317, 1318, 1323, and 1938) 1 week following infection and the serum of a sixth (sheep No. 1321) became positive 2 weeks after infection. The remaining 3 animals (sheep Nos. 1150, 1324, and 1970) developed positive neutralisation indices on or before the fourth week after infection. In 6 of the animals (sheep Nos. 1315, 1318, 1321, 1324, 1938, and 1970, those examined) the positive neutralisation titres persisted more or less unchanged until the end of the periods of observation which varied from 28 to 34 weeks. In general, the appearance of complement-fixing antibody was simultaneous with or followed that of neutralising antibody but in 2 instances (sheep Nos. 1324, and 1970) it preceded it.

Of the vaccinated sheep which took the disease after subcutaneous challenge and were regarded as convalescent, 2 were negative and the remainder positive by the complement-fixation test 8 weeks after initiating infection (table 23). Titres varied from 1 in 2 to 1 in 64. That all of these sheep were immune to louping-ill was established by the fact that they had survived a lethal intracerebral inoculation of virus given 2 weeks after subcutaneous challenge. The neutralisation indices of the same sera also verified immunity.

There were 2 instances of non-specific fixation amongst the convalescent sera (the samples from sheep No.1970 taken at the twentieth and twenty eighth week after infection) both/

Table 23

Results of complement-fixation tests with the convalescent sera of seven vaccinated sheep which took the disease on subcutaneous challenge with louping-ill virus, and freeze-dried benzol-extracted louping-ill and normal mouse brain antigens. The sera were drawn eight weeks after challenge; the neutralisation index of each serum is shown.

Antigen	Serum									
	Number	Dilution								Neutralisation index
		1:2	1:4	1:8	1:16	1:32	1:64	1:128	1:256	
L.I.*	2360	0	0	0	0	0	0	0	0	7,943
N■		0	0	0	-	-	-	-	-	
L.I.	2361	2	1	±	0	0	0	0	0	5,012
N		0	0	0	-	-	-	-	-	
L.I.	2362	4	4	4	4	3	2	±	0	15,850
N		0	0	0	-	-	-	-	-	
L.I.	2363	1	±	0	0	0	0	0	0	1,000
N		0	0	0	-	-	-	-	-	
L.I.	2364	2	2	±	0	0	0	0	0	501
N		0	0	0	-	-	-	-	-	
L.I.	2365	3	3	2	0	0	0	0	0	5,012
N		0	0	0	-	-	-	-	-	
L.I.	2366	1	2	2	±	0	0	0	0	794
N		0	0	0	-	-	-	-	-	
L.I.	Immune	4	4	4	4	4	3	1	0	10,000
N	control	0	0	0	-	-	-	-	-	
L.I.	Normal	0	0	0	0	-	-	-	-	
N	control	0	0	0	0	-	-	-	-	

Key as in table 8

* Louping ill

■ Normal

both of which were attributable to the anticomplementary action of the 2 specimens of serum, (table 22, test 9).

(ii) Natural convalescent sera

Twelve sheep sera submitted from the field (established as being convalescent by the neutralisation test) were tested for complement-fixing antibody (table 24). At what stages these sera were drawn in relation to the infections is not known; however, only 6 proved to be positive by the complement-fixation test. Titres varied from 1 in 2 to 1 in 16. Non-specific fixation was absent.

Table 24

Results of complement-fixation test with louping-ill convalescent sheep sera following natural infection and freeze-dried benzol-extracted louping-ill and normal mouse brain antigens. The times at which the sera were drawn in relation to the onset of the disease are not known. The neutralisation index of each serum is shown.

Antigen	Serum									
	Number	Dilution								Neutralisation index
		1:2	1:4	1:8	1:16	1:32	1:64	1:128	1:256	
L.I.*	DB7591- 2	½	2	2	±	0	0	0	-	100
N■		0	0	0	-	-	-	-	-	
L.I.	" - 3	3	4	4	3	1	0	0	-	316
N		0	0	0	-	-	-	-	-	
L.I.	" - 4	0	0	0	0	0	0	0	-	100
N		0	0	0	-	-	-	-	-	
L.I.	" - 8	4	4	4	±	0	0	0	-	100
N		0	0	0	-	-	-	-	-	
L.I.	DB7681	2	3	2	1	0	0	0	-	>3162
N		0	0	0	-	-	-	-	-	
L.I.	DB7686	2	3	3	±	0	0	0	-	178
N		0	0	0	-	-	-	-	-	
L.I.	DB7689	1	1	±	0	0	0	0	-	562
N		0	0	0	-	-	-	-	-	
L.I.	DB7603- 9	0	0	0	0	0	0	0	-	> 316
N		0	0	0	-	-	-	-	-	
L.I.	" -11	2	1	±	0	0	0	0	-	> 316
N		0	0	0	-	-	-	-	-	
L.I.	" -13	0	0	0	0	0	0	0	-	> 316
N		0	0	0	-	-	-	-	-	
L.I.	" -14	±	±	0	0	0	0	0	-	> 316
N		0	0	0	-	-	-	-	-	
L.I.	" -15	0	0	0	0	0	0	0	-	> 316
N		0	0	0	-	-	-	-	-	
L.I.	Immune	4	4	4	4	4	4	4	4	10,000
N	control	0	0	0	-	-	-	-	-	
L.I.	Normal	1	1	0	0	0	-	-	-	
N	control	0	0	0	0	0	-	-	-	

Key as in table 8.

* Louping ill.

■ Normal.

- Not done.

DISCUSSION

When adapting the complement-fixation test to a new viral disease it is usual to prepare an antigen rich in virus and antisera with high levels of antibody. At the same time, care must be taken to avoid anti-complementary action and non-specific fixation. In the present work, viable homologous brain virus was used to produce hyperimmune serum in sheep and the resulting neutralising antibody levels were comparable with those obtained by Wilson (1944) using a similar antigen. It might be expected that sheep repeatedly inoculated with homologous brain material would succumb to allergic encephalitis but, in fact, out of 18 only one died from this condition. It was, therefore, unnecessary to try the use of viable avian tissue culture virus shown by Wilson (1944) to be just as effective as homologous brain virus. Since clear evidence was obtained that the amount of complement-fixing antibody fell appreciably soon after the last immunising inoculation, the sheep had to be either sacrificed and bled out and the serum stored or they had to be given frequent booster doses of antigen so that sera of high titre could be withdrawn when required.

The complement-fixation test for viral agents is often unsatisfactory because of the difficulty of preparing a suitable antigen. The principal obstacle lies in the separation of the virus antigen from the antigen of the host tissues, a feature which must be controlled by the inclusion of an antigen consisting of an extract of normal tissues. In the present work, two sources of virus were used as antigen. Louping-ill virus propagated in the central nervous system of/

mice represents a source of virus of high titre which is stable for long periods when stored at -28°C. Mouse brain antigen was prepared after the freezing and thawing method of Casals and Palacios (1941). Such antigen was not anti-complementary. The potency of the mouse brain antigens was determined by "checkerboard" titrations with at least two hyperimmune sera. This led to the eventual selection of a 10 per cent. dilution of tissue for routine preparation of all mouse brain antigens. Casals and Palacios (1941) favoured centrifugation rather than filtration for separating virus mechanically from tissue components and they used a speed of 7,000 R.P.M. for 1 hour. In the present investigation, it was found that little or no benefit accrued, in terms of abatement of non-specific fixation, when the R.C.F. was raised above 5,750. On the other hand, ultra-centrifugation (a R.C.F. of approximately 81,430 for thirty minutes, or triple ultra-centrifugation at a R.C.F. of approximately 36,190 for thirty minutes on each occasion) resulted in a considerable reduction in the specific complement-binding potency of the antigen. Antigen boiled for 20 minutes lost its antigenicity completely. It was in fact found that, so far as sheep are concerned, the freeze-thaw method of preparing mouse brain antigen is only partially satisfactory in giving completely specific results. It was, however, found that antigen prepared by extracting freeze-dried mouse brain with benzol reacted non-specifically with fewer sera than antigen freeze-thawed or antigen freeze-dried and extracted with ether or acetone, if extraction was done by manual agitation. None of these antigens was anti-complementary./

Benzol-extracted antigen gave completely specific reactions with sheep sera when the process of extraction was improved as could be done by mechanical agitation with a magnetic stirrer. In this case the R.C.F. employed in removing the extraneous tissue from the aqueous antigen was the same as that employed when preparing freeze-thawed antigen.

The superiority of freeze-dried benzol-extracted mouse brain antigen over that which is freeze-thawed, so far as the elimination of fallacious fixation is concerned, must be attributed to the solvent action of benzol on the lipids in the tissues since, in all other respects, the antigens differed only slightly. There was some evidence, also, that the specific complement-binding potency of freeze-thawed antigen was only half that of freeze-dried extracted antigen as shown when both antigens were prepared from the same brain pool and tested simultaneously with the same hyperimmune serum. As this higher antigenicity was common to all freeze-dried antigens, irrespective of the solvent used, it may be assumed that freeze-drying <u>per se</u> is more effective in the release of intracellular complement-binding antigen than is freeze-thawing.

Although not representing as rich a source of virus as infected mouse brain, tissue culture of whole chick embryo yields virus in considerable quantity. Such virus used as a complement-fixing antigen was, however, very anti-complementary until the chick embryos were decapitated, thereby freeing the culture of a proportionally large quantity of brain tissue. This appeared to make no difference

to/

to the propagation of virus in culture. Such nutrient media as Tyrode's solution and Hanks' balanced salt solution both containing 10 per cent. sheep serum, Synthetic Mixture No. 199 and Synthetic Mixture No. 199 incorporating 2 per cent. normal horse serum used for the tissue culture, gave no more virus than did Tyrode's solution alone. Similarly, culture of chick chorio-allantois was no better for propagating virus than was chick embryo. The mean yield of virus from all tissue cultures was $10^{-4.7}$. However, in complement-fixation tests with hyperimmune sheep sera, such antigens, even in strengths greater than the mean stated, failed to fix complement, whereas specific fixation in high titre was obtained in parallel tests with freeze-thawed mouse brain antigen. Since the identity of the agent grown in tissue culture was established by neutralisation with louping-ill hyperimmune sheep serum, it can be concluded that failure of culture virus as a complement-fixing antigen was the result of an insufficiency of virus.

In complement-fixation tests with sheep sera and freeze-thawed antigen, it was necessary to minimise or eliminate, from the former, the properties contributing to non-specific fixation. The heating of sera to 65°C. for 20 minutes and the dilution of such sera to 1 in 4, removed this undesirable effect in most cases, without alteration of the specific titres of immune sera treated in this manner. Sera were rarely, if ever, anti-complementary even when diluted 1 in 2. Heating to 65°C. was regarded as the optimum temperature for this purpose since heating to 68°C., for the/

same period, lowered specific titres appreciably. Sera heated to 65°C. never gave non-specific reactions with the finally developed freeze-dried extracted brain antigen, even at a dilution of 1 in 2. The effect of being able to use serum diluted 1 in 2 instead of 1 in 4, in conjunction with the increased complement-binding potency of freese-dried extracted brain antigen, was to give a test which was significantly more sensitive than that with freeze-thawed brain antigen.

The infection of sheep by the subcutaneous inoculation of louping-ill virus,in order to study complement-fixing and neutralising antibodies in sera from animals in the acute phase of the disease and in convalescence,is not without danger to the sheep. For instance, Gordon et al. (1932) attempted, by means of subcutaneous titration of virus in sheep, to find a suitable dose which would reliably produce a mild infection and subsequent immunity. In the field, however, they found that the selected dose of virus killed 33 of 50 sheep. The outcome of infection given subcutaneously to susceptible sheep is, therefore, unpredictable. In the present investigation, 1 of 10 susceptible sheep similarly infected, developed such marked clinical symptoms of louping-ill that it had to be destroyed in extremis but contrary to what might have been expected, it was not possible to demonstrate by mouse inoculations virus in the central nervous system of this sheep. The remaining 9 sheep survived the infection and were immune. Edward (1949) was also unable to recover virus from the brains of 11 of 30 sheep (37 per cent.) which died of the disease after infection by the/

subcutaneous route followed 3 days later by a traumatising dose of sterile starch intracranially. If the absence of virus in the central nervous system is a feature of the natural disease in sheep, as these results may suggest, then diagnosis of the disease in the field by the isolation of virus from sheep brain, as generally examined, could be inaccurate.

There were several indications that the 9 sheep which survived inoculation with live virus, in the present investigation, became infected. All showed well defined febrile reactions, and viraemia was readily demonstrated in several. Also, serum neutralising antibody to louping-ill appeared and attained titres indicative of infection when assessed in accordance with the standards recommended by the Neurotropic Virus Disease Commission of 1942 (Smadel, 1952). None of these phenomena, however, is a sure indication that the animal has in fact had the disease and recovered from it. This can only be proven by intracranial challenge. For whereas it is possible to induce the formation of louping-ill antibody in sheep, and even to provide immunity to subcutaneous infection, by inoculation with dead virus, immunity of the central nervous system to intracranial challenge is only conferred by recovery from an actual attack of the disease (Gordon 1934; Wilson 1944). In this work, 2 of the animals under reference and 7 other sheep infected (in a similar manner) and immune on the basis of the serum neutralisation test, survived intracerebral challenge with live virus while 2 sheep, susceptible by the same test, and similarly challenged, were destroyed <u>in extremis</u> with the disease. It was assumed/

from the foregoing that the absence of neutralising antibody and its induction to recognised levels by experimental infection are satisfactory proof of susceptibility and convalescence, in the same sheep.

Titres of convalescent sera tested with the finally developed freeze-dried benzol-extracted mouse brain antigen were usually considerably lower than those of freshly prepared hyperimmune sera, and fixation was often partial (between 2 and 3) throughout the positive tubes or occasionally weak, occurring only in serum diluted 1 in 2. With the exception of the anti-complementary action of 2 samples of one serum, all results were completely specific as determined by corresponding tests with normal brain antigen. With these findings and the confirmation of convalescence as previously discussed, borderline titres, 2 or more fixation with serum diluted 1 in 2, must, in these instances, be regarded as signifying recovery from the disease. Under less exacting conditions, however, such as may be expected in a routine diagnostic laboratory, it is doubtful whether fixation in the first tube(1 in 2 dilution of serum) could by itself be considered as being significant. Sequential examination of the sera from 9 sheep before experimental infection, during the disease, and after recovery, showed that the appearance, titre, and persistence of complement-fixing antibody in the circulation varied considerably between individuals. The initial appearance of significant quantities of antibody was between the second and sixth weeks following infection and, invariably, such titres were the highest attained during convalescence. Maximal titres were maintained/

for a variable period after which they declined and disappeared altogether in under 21 weeks in more than 40 per cent. of the sheep. Since antibody appeared 2 to 6 weeks after infection and declined, in one instance, in under 10 weeks after infection, it was concluded that the optimum period for securing serum during convalescence, for the purpose of demonstrating complement-fixing antibody, is between the sixth and tenth week after infection or, approximately, 4 to 9 weeks after the clinical recovery of the sheep. When, however, another group of convalescent sheep was tested, 8 weeks after being given the disease, 2 were negative, 1 was borderline (2 fixation with serum diluted 1 in 2) and 4 were positive. As this group of animals was indeed convalescent (immune to intracranial challenge with live virus) then one cannot exclude the possibility that, in the 2 sheep which were nagative to the complement-fixation test, complement-fixing antibody may never have developed. The inconsistency of complement-fixing antibody was even more marked when sera from 12 field cases of the disease in sheep were examined. Although all of the sera were shown to possess neutralising antibody commensurate with infection (when compared with a serum from an uninfected sheep), only 50 per cent. showed complement-fixing titres. When these sera were drawn, in relation to the onset of the disease, is not known; however, the insufficiency of history accompanying sera submitted from the field is the rule rather than the exception.

It is evident from the foregoing, that while a completely/

specific complement-fixation test has been developed for louping-ill in sheep, with sera heated to 65°C. and freeze-dried mouse brain antigen (extracted with benzol by mechanical agitation and centrifuged at a R.C.F. of 5750 after aqueous rehydration), the test appears to be of little practical value in establishing the convalescent state since, the appearance, titre, and stability of complement-fixing antibody, induced by infection varies widely between individuals. Susceptible sheep were always negative to the test, but immune sheep were not always positive on account of the delayed appearance, borderline titres, and rapid decline and disappearance of antibody. In contrast, in the same sequential examinations, for neutralising antibody, positive titres were recorded from 1 to 4 weeks after infection or by the third week, approximately, after clinical recovery. Further, neutralising antibody was stable over a long period.

It was in the light of the above findings that attention was turned to seeking an alternative neutralisation test which was not dependent on the death of mice or chick embryos.

SUMMARY

1. Supernatant fluid, derived from louping-ill infected mouse brains which are freeze-thawed and then centrifuged as an aqueous emulsion, gives good fixation with freshly prepared hyperimmune sheep sera heated to 65°C. for 20 minutes but reacts non-specifically with a proportion of normal sheep sera similarly heated. The heating of sera in excess of this temperature lowers specific fixation while ultra-centrifugation of antigen lowers specific complement-binding potency without reducing the constituents responsible for non-specific fixation. Boiling of the antigen destroys antigenicity altogether.

2. Supernatant fluid, derived from benzol-extracted freeze-dried louping-ill infected mouse brains after rehydration and centrifugation, is of superior complement-binding potency to freeze-thawed antigen and does not react non-specifically with sheep sera heated to 65°C., provided extraction is rendered thorough by mechanical agitation.

3. Avian tissue culture virus failed to fix complement in the presence of immune serum; this was attributed to an insufficiency of virus.

4. Titres of convalescent sera from sheep experimentally infected with the disease are usually considerably lower than those of hyperimmune sera and fixation is often partial (between 2 and 3) throughout the positive tubes or, not infrequently, borderline, than is occuring in the first tube (serum diluted 1 in 2) only.

5. Complement-fixing antibody appears in sheep 2 to 6 weeks after they are experimentally infected; it persisted for/

only 6 weeks in one case and for less than 21 weeks in 40 per cent. of the sheep examined. In contrast, positive neutralisation titres appeared 1 to 4 weeks after infection and this antibody was stable over a long period.

6. Due to the delayed appearance, borderline titres, and rapid decline and disappearance of complement-fixing antibody in a high proportion of convalescent sheep, such animals are not always positive to the complement-fixation test. Thus, only 50 per cent. of sera from field cases of the disease, as diagnosed by the neutralisation test, did contain complement-fixing antibody. However, neutralising antibody is always present at accepted levels in the convalescent sheep from within 3 weeks of clinical recovery.

II. CYTOPATHOGENICITY IN PIG KIDNEY MONOLAYER TISSUE CULTURE

INTRODUCTION

Since Huang (1943) reported that the Western strain of equine encephalomyelitis virus could be titrated *in vitro* by its destructive action on chick embryo cells grown in culture, a number of viruses have been shown to be cytopathogenic for various cell cultures. Notable among these are poliomyelitis for human tissues (Enders, *et al.*,1949; Robbins, *et al.*, 1950), foot-and-mouth disease and vesicular stomatitis for pig and calf kidney respectively (Sellers, 1955) and infectious canine hepatitis for dog kidney (Cabasso, *et al.*, 1954).

Several modifications have been made to the original technique of titration. With the so called suspended-cell or suspended-fragment (Maitland) type culture, 3 methods are available, *viz*:- (1) histological examination of infected fragments; (2) the 'pH-differential test' in which infected tissues usually fail to maintain a metabolic rate, as measured by acid production, comparable to uninfected control tissue; and (3) inhibition of cell migration from infected tissue fragments removed from culture flasks to hanging drop preparations. The tube methods of culture of fixed tissue fragments afford direct microscopic examination of the live cell outgrowths *in situ*. Recently, Dulbecco (1952) developed an accurate macroscopic method of virus titration which involved the counting of plaques produced by the pathogenic action of the virus on susceptible cells grown as a monolayer/

monolayer in tissue culture. The monolayer was achieved by seeding culture vessels with a suspension of trypsin-dispersed cells. Youngner (1954) adapted the monolayer technique to tube-culture, an innovation which has all but rendered the culture of fixed tissue fragments obsolete.

The purpose of the following investigation was to attempt to find a cell which when grown in tissue culture and infected with louping-ill virus developed a specific lesion which would enable titration of the agent in vitro. For this purpose, kidney cells of 3 susceptible species namely, pig, sheep,and chick embryo were selected and the monolayer roller tube technique of culture adopted.

The method of culture of pig kidney was that made available by Sellers (1957) while, as far as is known, no previous description of sheep kidney culture exists. Trypsin-dispersion of these tissues was carried out according to the recommendations of Rappaport (1956). Chick embryo kidney was dispersed and grown as described by Buthala and Mathews (1957).

MATERIALS AND METHODS

1. Virus

The louping-ill virus used was derived from mouse brain. The history of the virus, the method of storing, handling and titration in mice were the same as described in Section 1.

11. Technique of monolayer tissue culture

A. Glassware and apparatus

(i) **Glassware**

(i) Glassware

'Pyrex' glassware was used throughout except for pipettes and cover-glasses. Glassware was immersed in an inorganic detergent* in enamel containers and boiled for 20 minutes. When cool, a wa ter hose was inserted to the bottom of the containers and the detergent was floated away. The glassware was washed in 8 rinses of running tap water. It was then immersed in a N/40,000 solution of hydrochloric acid for 20 minutes followed by 8 rinses in running tap water and finally 3 rinses in running glass-distilled water. The dry glassware was sterilised at 160°C. for 1 hour in a dry air oven. Coverglasses (0.63 x 2.22 cm.) were boiled twice in glass-distilled water and passed individually through ether and 2 changes of absolute alcohol; they were sterilised singly within roller tubes. As routine, cultures were prepared in Roux flasks (12 x 20 cm.) and/or in roller tubes (1.6 x 15cm.).

(ii) Stoppers

White 'non-toxic' rubber stoppers were used. These were boiled twice in glass-distilled water prior to sterilisation at 20 lb. pressure for 20 minutes.

(iii) Rotary drum

Following initial stationary incubation, all of the tube-cultures/

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* Sodium hexametaphosphate, 40 gm.; sodium metasilicate (technical), 360 gm.; water, 4 litres. The solution was allowed to stand overnight, then filtered and made up to volume with water. Before use, the solution was diluted 1 : 100 in water.

tube-cultures were placed in a roller drum mounted in a bacteriological incubator. The drum was inclined sufficiently to prevent the nutrient medium from coming in contact with the stoppers and was rotated at 12 to 14 revolutions per hour. All incubation was carried out at 35° to 36°C.

B. Constituents of the culture

(i) Earle's saline

Earle's saline (Earle, 1943) was prepared as follows:- NaCl*, 6.80 gm.; KCl, 0.40 gm.; $CaCl_2.2H_2O$, 0.20 gm.; $MgSO_4.7H_2O$, 0.20 gm.; $NaH_2PO_4.H_2O$, 0.125 gm.; $NaHCO_3$, 2.20 gm.; dextrose, 1.0 gm.; glass-distilled water 1000 ml. The $NaHCO_3$ was dissolved in 200 ml. of water and the other ingredients in the remaining 800 ml. Each solution was sterilised by filtration through a 5/3 sintered-glass filter under negative pressure and the two mixed aseptically and stored tightly stoppered in 500 ml. amounts at 4°C. The pH of the solution was unadjusted.

(ii) Hanks' balanced salt solution

Hanks' balanced salt solution was prepared after the method of Weller, et al. (1952) as described in Section 1.

(iii) Phosphate-buffered saline

Phosphate-buffered saline was prepared in 3 separate solutions thus:-

(a)/

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* Salts of analytical reagent grade (Analar) were employed throughout.

(a) NaCl, 8.0 gm.; KCl, 0.2 gm.; $Na_2HPO_4.12H_2O$, 2.9 gm.; KH_2PO_4, 0.2 gm.; glass-distilled water, 800 ml.; (b) $CaCl_2.6H_2O$, 0.2 gm.; glass-distilled water, 100 ml.; and (c) $MgCl_2.6H_2O$, 0.1 gm.; glass-distilled water, 100 ml. These solutions were autoclaved at 10 lb. pressure for 10 minutes and mixed aseptically when cool. The pH was 7.5.

(iv) Lactalbumen hydrolysate (enzymatic)

Lactalbumen hydrolysate was prepared as a 5.0 per cent. solution in Hanks' balanced salt solution without sodium bicarbonate and was sterilised by autoclaving at 10 lb. pressure for 10 minutes. The solution was stored at 4°C. and, if a precipitate formed, it was brought to the boil immediately before use.

(v) Yeast extract

A 0.1 per cent. solution of yeast extract ('Difco') was made in Hanks' balanced salt solution without sodium bicarbonate. The solution was autoclaved at 10 lb. pressure for 10 minutes and stored at 4°C.

(vi) Normal sera

(a) Cattle

Cattle blood was collected at the abattoir, allowed to clot, and the serum removed and sterilised by filtration through a G.S. Seitz pad. The serum from each animal was kept separate and storage was carried out in the frozen state at -28°C. Only sera free from louping-ill antibody (as determined by mouse titration) were used. Serum was inactivated/

inactivated at 56°C. for 30 minutes before being incorporated in nutrient media.

(b) Sheep

Sheep serum was prepared in an identical manner from blood drawn from an animal known to be free from louping-ill antibody.

(vii) Tissues

(a) Pig kidney

Kidneys were secured from freshly slaughtered animals weighing approximately 225 lbs. live weight. A minimum of 2 organs, each from different animals, were prepared at any one time as a marked variation in the growth of kidney from different pigs was sometimes encountered.

(b) Sheep kidney

Kidneys were collected fresh from normal sheep sacrificed at the Moredun Institute.

(c) Chick embryo kidney

Whole chick embryo kidney was taken aseptically from embryos incubated at 38° to 39°C. for 18 to 20 days.

C. Composition of nutrient media.

(i) Medium for pig cells

Earle's saline	72	per cent.
Lactalbumen Hydrolysate	9	" "
Yeast extract	9	" "
Normal cattle serum	10	" "

Phenol red, as prepared for Hanks' balanced salt solution was/

was diluted to 2 per cent. strength in distilled water and 0.1 ml. added to each 10 ml. of Earle's saline and cattle serum in the medium. Antibiotics were added to give the following concentrations per ml. of medium:- penicillin, 200 units; streptomycin, 0.2 milligram; and 'Mycostatin,' 80 units (Wigmore and Henderson, 1955). The pH of the medium was usually about 7.4 and it was used at this value. When on occasions the medium was slightly more acid, it was permitted to lose carbon dioxide through a cotton stopper until the desired pH was obtained.

(ii) Medium for sheep cells

The medium used in sheep cell culture was identical with that described for pig except that homologous serum replaced that of cattle.

(iii) Medium for chick embryo cells

Hanks' balanced salt solution	97.5 per cent.
Lactalbumen hydrolysate	0.5 " "
Normal cattle serum	2.0 " "

Antibiotics were utilised as before and the pH was adjusted to 7.6 to 7.8 by adding sodium bicarbonate as a sterile 1.4 per cent. solution.

D. General procedure for maintaining sterile technique

Whenever practicable, manipulations were performed beneath a 'hood' which was wiped out with lysol prior to use. Mycotic, yeast, and bacterial contaminants were rarely encountered in cultures incorporating antibiotics in the nutrient medium.

E. Preparation of cultures

(i) Pig kidney

The tissue of each pig was kept separately throughout until ready for subculture. Kidneys were decapsulated and the cortices cut into pieces 0.5 cm. cube. These were put into Erlenmeyer flasks at the rate of about ½ a kidney to a 200 ml. flask and were washed with several changes of warm phosphate-buffered saline until the latter was clear. The tissues were then covered with warm 0.3 per cent. trypsin* solution, and incubated at 37°C. for 1 hour. At the end of this period, the contents of the flasks were agitated by means of magnetic stirrers for 7 minutes at a rate just below the point of foaming. The dispersed cells were decanted and temporarily stored at 4°C. Fresh warm trypsin solution was added to the flasks and the stirring and decanting carried out as before; the process was repeated until sufficient cells were obtained.

The cells were recovered from the chilled trypsin by horizontal centrifugation at a R.C.F. of approximately 11 for 30 minutes. After one wash in warm phosphate-buffered saline followed by centrifugation as before, they were resuspended in nutrient medium and filtered through 2 layers of sterile surgical gauze. The suspension was finally standardised by cell count (Rappaport, 1956) at 8×10^5 cells per ml. of medium.

Primary cultures were prepared by introducing 60 ml. of cell/

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* Trypsin ('Difco'1:250) was prepared in phosphate-buffered saline free from calcium salt and was sterilised by filtration through a G.S. Seitz pad.

cell suspension into Roux flasks. These were tightly stoppered and incubated on their sides for 6 days with one change of medium on the third day. By this method almost confluent monolayers, predominantly of epithelial cells with some fibroblasts, were formed. On the sixth day the monolayers were washed with warm phosphate-buffered saline free from calcium and magnesium salts and were dislodged from the glass by the action of versene*. Twenty ml. of freshly prepared versene were used per flask and the cells came free after about 20 minutes incubation at 37°C. The versene-cell suspensions were pooled and centrifuged as before. The cells were resuspended in fresh medium, standardised at 8 x 10^5 cells per ml. and subcultured into tubes containing coverglasses at the rate of 0.5 ml. each by means of a pipette or automatic syringe. Tubes were tightly stoppered and incubated at an angle so that the cell suspension overlayed the coverglasses. Cultures were ready for inoculation by the second to third day when continuous monolayers of epithelial cells were formed; further incubation was carried out in a rotary drum.

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* Versene (disodium salt of ethylene-diamine-tetra-acetic acid) was used as a 0.02 per cent. solution in phosphate-buffered saline free from calcium and magnesium salts. The preparation was sterilised at 10 lbs. pressure for 10 minutes.

(ii) Sheep kidney

The method of preparing monolayer cultures from sheep kidney was the same as for pig except that cells were sewn directly into tubes by introducing into each 0.5 ml. of suspension containing 4 x 10^5 cells per ml. of medium. Cultures were ready for inoculation on about the fourth day of stationary incubation when continuous monolayers of epithelial cells were formed. At this stage the tube-cultures were placed in a rotary drum.

(iii) Chick embryo kidney

Whole kidneys from 4 to 6 embryos were washed in 30 ml. of Hanks' balanced salt solution and digested in 10 ml. of 0.5 per cent. trypsin in Hanks'. The digestion vessel was shaken vigorously for about 2 minutes to commence dispersion and this was repeated at 5 minute intervals over a period of 20 minutes during which the vessel was immersed in a bath at 37°C. The dispersed cells were washed twice in Hanks' balanced salt solution and resuspended in nutrient medium to contain 4 x 10^5 cells per ml. of medium. Tubes were sewn directly with 0.5 ml. of cell suspension and were ready for inoculation and rolling after 48 hours incubation. The monolayer comprised epithelial cells and fibroblasts.

F. Maintenance of cultures

The nutrient medium in all cultures was removed and renewed with freshly prepared medium every 3 to 4 days. In the case of tube-cultures the volume of nutrient medium was always/

always increased from 0.5 ml. to 1.0 ml. on the occasion of the first replacement of medium. The progress of cell growth was followed under low power magnification.

No special study was carried out in connection with the longevity of cultures. On one occasion, however, pig monolayers were maintained after initial subculture for as long as 6 weeks without further subculture.

G. Inoculation of cultures

Cultures to be inoculated were drained as completely as possible of medium by means of a suction pump and sterile capillary pipette. The inoculum was added to a pool of nutrient medium 9 times its volume and 1.0 ml. of the inoculum-medium mixture introduced into each tube-culture. The volume of inoculum introduced into each tube was therefore 0.1 ml. in 0.9 ml. of nutrient medium and unless otherwise stated, the dilution of inoculum was always that added to the pool of nutrient medium. The interval between mixing the inoculum and nutrient medium and overlaying the monolayers with the mixture was kept to a minimum.

H. Sterility test

As routine, medium from each tube-culture was plated onto blood agar at the termination of its use. Bacteriological cultures were incubated for 2 days at 37°C. followed by 2 days at room temperature. Contaminants were rarely encountered.

III. Fixing and staining of coverglass monolayer preparations.

Medium was removed as completely as possible from tubes/

tubes containing coverglasses with monolayers for permanent mounting. The monolayers were washed once in Earle's saline pH7.4 and fixed for 1 minute within the tubes with methyl alcohol. The dry fixed monolayers, still within the tubes, were then stained overnight with aqueous Giemsa*. On the following day the coverglasses were removed from the tubes, rinsed in distilled water and differentiated in 0.25 per cent. colophonium resin in 95 per cent. alcohol. They were then washed in tap water and rapidly dehydrated in acetone, acetone-xylol, and Xylol and mounted in D.P.X. (Kirkpatrick and Lendrum, 1941). Coverglass preparations intended for macroscopic observation, as for example those in figure 3, were very briefly differentiated in tap water only.

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* Giemsa's stain (Hopkin and Williams Ltd., Essex, England) 1 drop to 1.0 to 1.5 ml. of water.

EXPERIMENTAL RESULTS

1. The effect of virus on kidney cells of pig, sheep, and chick embryo grown in culture.

Tube-cultures of each tissue were divided into 3 equal groups of at least 12 tubes and the cultures treated as follows upon removal of the medium:-

Group 1: Inoculated with 1 in 10 dilution of louping-ill mouse brain virus (approximate mouse titre of $10^{-7.7}$), that is 0.1 ml. 1 in 10 dilution of virus in 0.9 ml. nutrient medium;

Group 2: Inoculated with 1 in 10 dilution of normal mouse brain;

Group 3: Inoculated with virus diluent, that is 0.1 ml. saline-serum in 0.9 ml. nutrient medium.

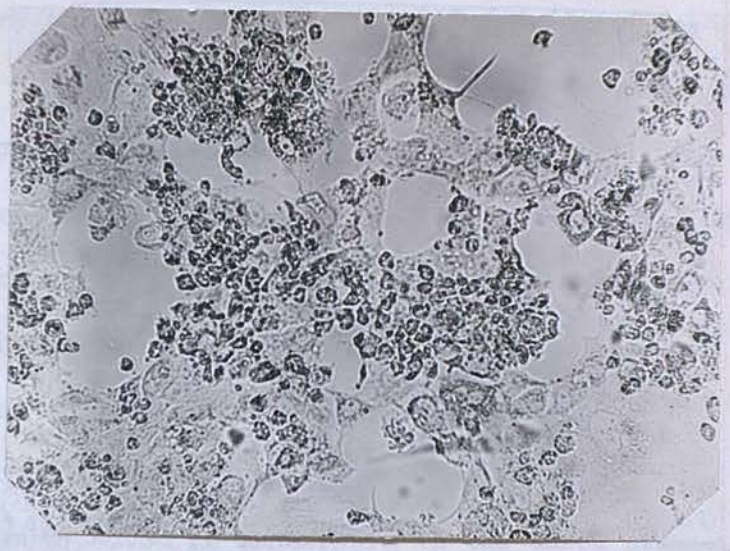
The cultures were incubated for 6 days and were examined daily under low power magnification for any obvious cytopathogenic effect.

A. Pig cells

On the second to third day of incubation the cells in cultures containing virus (group 1) commenced to deviate in microscopic appearance from those in the controls (groups 2 and 3). The former cells turned granular, the nuclei became swollen, and the cells rounded-up and appeared to agglutinate (similar to figure 1 (a)). The pathogenic effect increased in severity until by the/

Figure 1

The cytopathogenic effect of louping-ill virus in pig kidney monolayer tissue culture (unfixed and unstained).



(a) Cytopathogenicity produced in pig kidney monolayer tissue culture by approximately 300 CPD_{50} of louping-ill mouse brain virus in the presence of an equal volume of normal sheep serum diluted 1 in 100. x 110.

(b) Normal pig kidney monolayer 'protected' from the same amount of virus by an equal volume of louping-ill hyperimmune sheep serum diluted 1 in 100. The source and age of the cells as well as the period of incubation with virus were identical with (a). x 110.

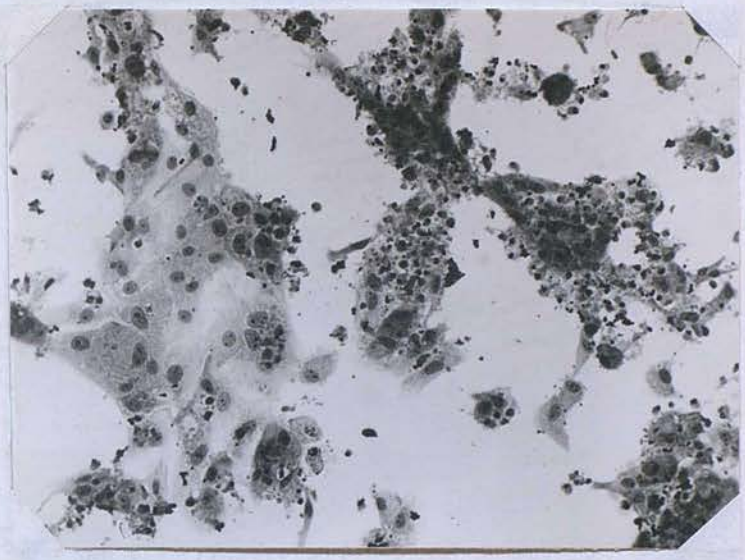
the fifth day there was almost total cell necrosis and destruction of the monolayers. The cells and monolayers of cultures in groups 2 and 3 remained normal (similar to figure 1 (b)). Fixed coverglass preparations of affected cells stained with Giemsa's (figure 2 (a)), showed shrinking, pyknosis, disintegration, and fragmentation of the monolayers; these effects were absent from similar preparations of monolayers amongst the controls (figure 2 (b) and (c)). Figure 3 attempts by means of a minimum of differentiation of the stained coverglasses, to illustrate the macroscopic sequential changes which occurred in monolayers in the three groups. It will be seen that some change in the staining qualities of monolayers in group 1 was noticeable on the second day after inoculation of virus and that thereafter a rapid diminution of the monolayers occurred. In contrast, those of groups 2 and 3 remained intact. Mice inoculated intracranially with pooled medium from group 1 cultures harvested on the fifth or sixth day died of typical louping-ill while medium from groups 2 and 3 caused no illness or death in mice.

B. Sheep and chick embryo cells

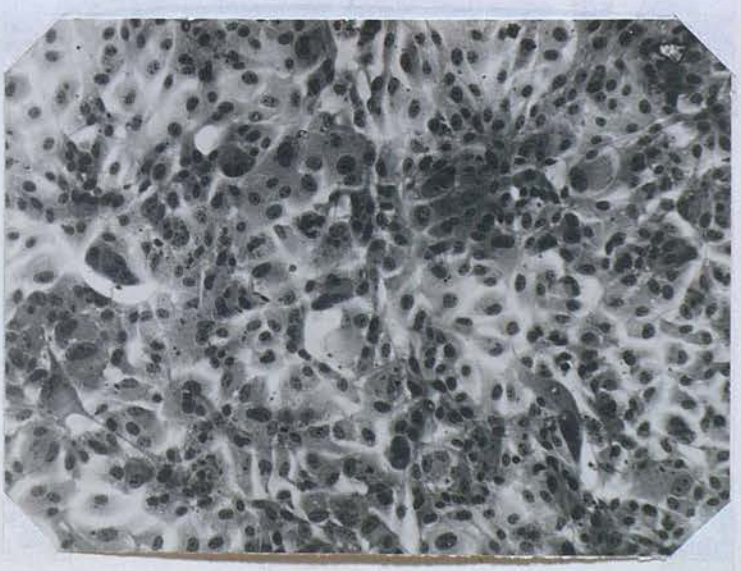
Virus had no effect on the appearance of sheep or chick embryo cells examined microscopically over a period of 5 to 6 days as live cultures. Mice inoculated intracranially with medium harvested from group 1 cultures of both types of cells at the termination of the period of observation died of typical louping-ill. In the circumstances, sheep and chick embryo cultures were abandoned so that studies on those of the pig could be intensified.

Figure 2

The cytopathogenic effect of louping-ill virus in pig kidney monolayer tissue culture (fixed and stained).

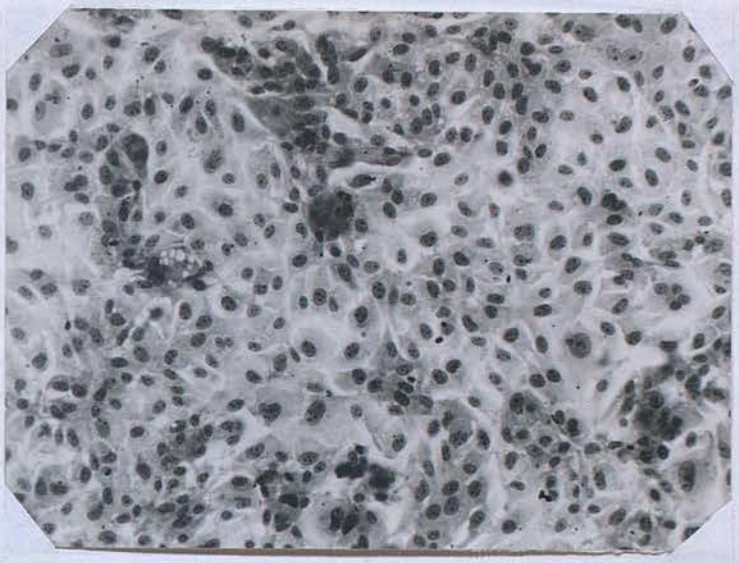


(a) Cytopathogenicity produced in pig kidney monolayer tissue culture after 3 days incubation with 1 in 10 dilution of louping-ill mouse brain virus (approximate mouse titre of $10^{-7.7}$). Giemsa's stain, x 110.



(b) Normal pig kidney monolayer after 3 days incubation with 1 in 10 dilution of normal mouse brain. Giemsa's stain, x 110.

Figure 2 contd.



(c) Normal pig kidney monolayer after 3 days incubation with virus diluent (saline-serum). The source and age of the cells and the volume of inoculum were the same as in (a) and (b). Giemsa's stain, x 110.

Figure 3

The macroscopic sequential effect of 1 in 10 louping-ill mouse brain virus (approximate mouse titre of $10^{-7.7}$) on pig kidney monolayer tissue grown on coverglasses in individual tube-cultures. The source and age of the cells and volume of inoculum were the same in the control cultures. Giemsa's stain; approximately one half the actual size.

Incubation in days	Inoculum		
	Virus diluent	1 in 10 normal mouse brain	1 in 10 louping-ill mouse brain
1			
2			
3			
4			
5			

II. Multiplication of virus in pig kidney culture

Virus was passaged serially in culture by transferring pooled undiluted or diluted infected medium from one group of 4 to 6 tube-cultures to each of a group of newly prepared ones. Passage virus was harvested on the sixth day of incubation and titrated immediately in mice. By the fifth passage the original virus inoculum titre of 4×10^4 mouse LD_{50} per ml. was diluted 10^7 but yielded 3.9×10^7 mouse LD_{50} per ml. (table 1). Clearly, the cultures were supporting multiplication of the virus.

III. Titration of virus by its cytopathogenic effect in pig kidney cultures.

Titration of virus in culture on the basis of the presence or absence of cytopathogenicity was carried out by adding tenfold dilutions of mouse brain virus to groups of 5 tube-cultures. Results were read on the sixth day of incubation under low-power magnification and the 50 per cent. end point calculated as in mice. Mice were inoculated simultaneously with the identical virus dilutions. Tables 2 and 4 present the results of two such titrations of a mouse brain virus pool. One cytopathogenic dose $_{50}$ (CPD_{50}) was determined to be equivalent to 300 to 600 mouse LD_{50}.

IV. Neutralisation of the cytopathogenic effect in pig kidney culture with louping-ill hyperimmune sheep serum.

Neutralisation of the cytopathogenic effect in culture was carried out both by serum and virus dilution dilution/

Table 1. Multiplication of the virus of louping-ill passaged serially in pig kidney cultures

Passage number	Total days in culture	Cumulative dilution of original virus inoculum	Titre of virus in medium Mouse LD_{50}/ml.
	6		4×10^4
1	12	10^1	-
2	18	10^2	-
3	24	10^3	-
4	30	10^4	2×10^6
5	36	10^7	3.9×10^7

- Not done

Table 2. Titration of louping-ill mouse brain virus in pig kidney monolayer tissue culture on the basis of the presence or absence of cytopathogenicity. A titration done simultaneously in mice with the identical virus dilutions is also shown.

Test system	Virus										
	Inoculum in ml.	Log-dilution									Titre-log dilution 50 per cent. end point
		10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}	10^{-8}	10^{-9}	
Cultures	0.1	5/5*	5/5	5/5	5/5	5/5	2/5	0/5	0/5	0/5	$10^{-5.8}$ (=1CPD$_{50}$)
Mice	0.05	3/3■	4/4	4/4	4/4	4/4	4/4	4/4	2/4	0/4	$10^{-8.0}$ (=1LD$_{50}$)

* Numerator - number of tube-cultures showing cytopathogenic effect up to the sixth day after inoculation.
Denominator - number of tube-cultures inoculated.

■ Numerator - number of mice killed showing typical symptoms of louping-ill up to the fifteenth day after inoculation.
Denominator - number of mice inoculated.

dilution techniques. In the former method tenfold dilutions of sera were mixed with an equal volume of virus so that the inocula of serum-virus mixtures for cultures and mice contained approximately 300 CPD_{50} and 300 mouse LD_{50} of mouse brain virus respectively. Serum-virus mixtures were incubated *in vitro* for 2 hours at room temperature followed by $\frac{1}{2}$ to 1 hour at 4°C. prior to simultaneous inoculation into the test systems. Five tube-cultures were inoculated per dilution and the results were read on the sixth day. In the virus dilution technique tenfold dilutions of virus commencing at 1 in 5 were mixed with an equal volume of serum diluted 1 in 10. Serum-virus mixtures were incubated as before prior to inoculation into cultures and mice.

Tables 3 and 4 present the results of neutralisation tests by the serum and virus dilution methods respectively in which the same virus and sera were used. The marked inhibition of cytopathogenicity in both instances by the louping-ill hyperimmune sheep serum was regarded as proof of the specificity of the reaction.

Table 3. Neutralisation of the cytopathogenicity induced by louping-ill mouse brain virus in pig kidney monolayer tissue culture by specific hyperimmune sheep serum. Simultaneous neutralisation in mice inoculated intracranially is also shown.

Test system	Inoculum ml.	Quantity of virus	Sheep serum log dilution												Log dilution L.I.▲ hyperimmune serum which 'protected' 50 per cent. of test system
			Normal						Louping-ill hyperimmune						
			Undil.	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	Undil.	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	
Cultures	0.1	300 CPD_{50}	5/5*	5/5	5/5	5/5	5/5	5/5	0/5	0/5	0/5	0/5	5/5	5/5	$10^{-3.5}$
Mice	0.05	300 LD_{50}	4/4■	3/3	4/4	4/4	4/4	4/4	0/4	1/3	0/4	1/3	4/4	4/4	$10^{-3.0}$

*) Abbreviations as in table 2.
■)

▲ Louping-ill.

Table 4. Neutralisation of the cytopathogenicity induced by louping-ill mouse brain virus in pig kidney monolayer tissue culture by specific hyperimmune sheep serum. Simultaneous neutralisation in mice inoculated intracranially is also shown.

Test system	Inoculum ml.	Sheep serum 1:10	Virus-log dilution									Titre	
			10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}	10^{-8}	10^{-9}	Log dilution 50 per cent. end point	Neutralisation index
Cultures	0.1	Normal	5/5✱	5/5	5/5	5/5	3/5	2/5	0/5	0/5	0/5	$10^{-5.48}$	
		L.I.▲ hyperimmune	3/3	0/5	0/5	0/5	-	-	-	-	-	$10^{-1.5}$	10,000
Mice	0.05	Normal	-	-	-	-	-	4/4■	4/4	2/4	0/4	$10^{-8.0}$	
		L.I.▲ hyperimmune	-	-	3/4	2/4	1/4	0/3	-	-	-	$10^{-4.0}$	10,000

✱) Abbreviations as in table 2.
■)
▲ Louping-ill
- Not done

DISCUSSION

Evidence has been presented to show that tissue cultures of pig kidney support the growth of louping-ill virus and, in so doing, undergo severe degenerative changes leading to cell necrosis. The effect was sufficiently clear for the cultures to provide their own index of virus activity.

Monolayer tissue cultures of trypsin-dispersed pig kidney cells have not been widely used in virus research. Sellers (1955) and Bachrach et al. (1955) were among the first to do so both using this cell as host for foot-and-mouth disease virus. Sellers, utilising the method of Dulbecco and Vogt (1954), obtained monolayers consisting mainly of epithelial cells and some fibroblasts 4 to 8 days after initial seeding. The sheeting of cells was sufficiently confluent to permit the use of the plaque counting technique. Recently, Young et al. (1957) carried out an investigation into the development of procedures for the growth of cells freed from cortex of pig kidney by trypsin but concluded that their growth was unsatisfactory for continuous monolayers on glass. The difficulty of achieving confluent sheets was experienced in the present work; however, the problem was readily overcome by subculturing the cells grown from primary seeding (Sellers, 1957). Subcultured cells grew and sheeted rapidly and they appeared to be entirely epithelial.

The requisite for the development of a plaque is a high destructive activity of the virus on the host cells in culture. Although plaques induced by louping-ill virus in

pig/

pig cells were successfully demonstrated and inhibited with antiserum in a limited number of tests, the technique was generally unsatisfactory. On both occasions, the onset of non-specific degeneration of the agar overlayed monolayers (fifth day) coincided with the delayed development of plaques. The slow appearance of plaques was probably due to the low titre of virus used in the inoculum. It is possible that the technique could be improved by better methods of monolayer maintenance along with virus inocula of somewhat higher titres than those used in these tests, although a titre would be reached beyond which the coalescence of plaques would render the technique useless.

Titrations of virus and antiserum in tube-cultures on the basis of the presence or absence of virus cytopathogenicity were as accurate as titrations in mice. The procedure used in reading all tube-culture titrations was for the writer and a colleague to examine independently the unidentified cultures grouped by dilutions; the cultures of each dilution were examined in a fixed order so as to permit comparison of readings in individual tubes. The following shows that there was close similarity of results and substantiates the clear cut nature of the criterion of the technique:-/

technique:-

Titration	Titre-Examiner A.	Titre-Examiner B.
1	$10^{-5.8}$	$10^{-5.5}$
2	Nil	Nil
3	$10^{-3.5}$	$10^{-3.5}$
4	$10^{-5.4}$	$10^{-5.2}$
5	$10^{-1.5}$	$10^{-1.0}$

Although large numbers of tube-cultures can be rapidly examined microscopically it is probable that the technique could be adapted to the 'pH differential test' by replacing Earle's saline in the nutrient medium with a less highly buffered physiological salt solution such as Hanks'. Alternatively, it is possible that titrations could be carried out on the basis of the staining qualities of the monolayers to give a macroscopic effect similar in appearance to that shown in figure 3. For this purpose, a vital stain such as neutral red could be incorporated in the nutrient medium.

Since the completion of these experiments, the publication by Oker-Blom (1956) on the cytopathogenicity of louping-ill virus for HeLa cells has come to the attention of the writer. In this culture system the pathogenic changes were not clearly apparent until the fifth to seventh day.

Several workers have compared the sensitivity of the mouse in the titration of louping-ill virus and antibody to that of other test systems. Burnet (1936) found pock titration on the chorio-allantoic membrane of the egg to be about 10 times more sensitive while proportionally more virus was neutralised/

neutralised by antiserum. Wilson (1944) compared a Maitland-type tissue culture of chick embryo suspended in Tyrode's solution by making subinoculations of culture fluid intracranially into mice to detect the presence or absence of virus. Disregarding the increased chances of the larger inoculum in culture (2.0 ml. as opposed to 0.05 ml. in mice) containing an infectious unit of virus, the cultures were 0.25 to 25 times more sensitive and about 20 times more virus was neutralised. Similarly, titration based on the fatal infection of the chick embryo following yolk sac inoculation was 100 times more sensitive (Edward , 1947).

Although these alternative means of titrating the agent and its antiserum are all superior in sensitivity to intracranial titration in the mouse, it is true to say that the latter method has never been superseded in practice. Pock titration on the chorio-allantois is difficult. Lesions are frequently small and weak and often give rise to secondary foci; non-specific lesions on the membrane add to the difficulties. Titration in Maitland-type tissue culture is impracticable in the absence of any inherent index of virus activity. Titration by the death or survival of the chick embryo infected via the yolk sac poses the problem of candling large numbers of eggs and, more important, that of non-specific death of embryos.

By comparison, it would appear that titration by virus cytopathogenicity in tube-cultures of pig kidney cells is the least sensitive of the available test systems, 1 CPD_{50} representing/

representing 300 to 600 mouse LD_{50}. It should be borne in mind, however, that the practice adopted of reading titrations in culture on the sixth day following inoculation was purely an arbitrary one and that the sensitivity of the technique could certainly be enhanced by prolonging, by several days if necessary, the period of exposure of the cultures to virus. Such would be quite feasible from the point of view of the survival of the cultures in respect of non-specific degenerative changes. It is conceivable also, that the pig kidney culture is capable of propagating minute inocula of virus in sub-pathogenic titres, the detection of which would require sub-inoculation into fresh cultures.

The demonstration of the cytopathogenic effect of louping-ill virus on pig kidney monolayer tissue culture represents an important development in connection with research into the disease. Titration of virus and antiserum by this technique has the advantage of being both cheaper and faster than that in mice without any loss in accuracy. The need for a relatively simple inexpensive test for the identification of louping-ill infection in large numbers of animals has long been recognised. Problems such as surveys of sheep and cattle populations in retrospect diagnosis, the selection of susceptible stock for large scale vaccine trials, and the evaluation of the immune response to such vaccines have remained unresolved on account of the overwhelming cost of animal titration. It is felt that monolayer/

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Growth and Titration of Louping-ill Virus in Monolayer Tissue Culture of Pig Kidney

Rivers and Ward[1] reported the propagation of louping-ill virus in a Maitland-type culture of chick embryo tissue. More recently, Oker-Blom[2] has described a cytopathogenicity in HeLa cell culture induced by louping-ill which permitted titration of the virus. I have found a specific cytopathogenic effect by the same viral agent in monolayer culture of pig kidney.

The louping-ill virus was a strain isolated and maintained at the Moredun Institute and is used here as infected mouse brain. Virus dilutions were prepared in physiological saline (0·85 per cent sodium chloride) incorporating 10 per cent normal sheep serum, and titrations were carried out intracranially in four 3–5 week old white mice per dilution. Mice were observed for fifteen days following inoculation, and the 50 per cent end-point arrived at by the method of Reed and Muench[3]. The method of culture of pig kidney was that made available by Sellers

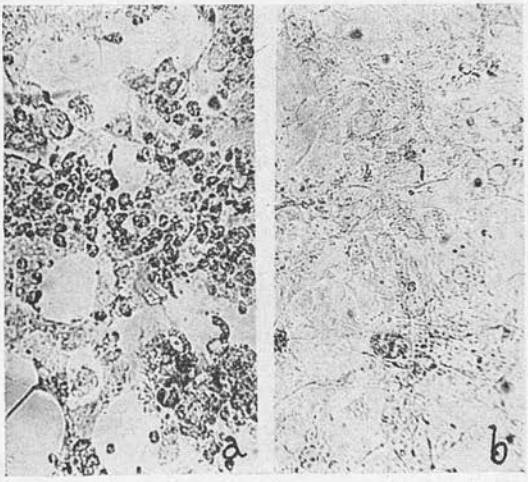

Fig. 1. (*a*) Cytopathogenicity produced in pig kidney monolayer tissue culture by approximately 300 *CPD*50 doses of louping-ill mouse brain virus in the presence of an equal volume of normal sheep serum diluted 1 in 100. (*b*) Normal pig kidney monolayer 'protected' from the same amount of virus by an equal volume of louping-ill hyperimmune sheep serum diluted 1 in 100. The source and age of the cells as well as the period of incubation with virus were identical with (*a*). × 110

culture was carried out both by serum and virus dilution techniques. The results of a test in which tenfold dilutions of sera were tested against approximately 300 *CPD*50 doses and 300 mouse *LD*50 doses of mouse brain virus in cultures and mice respectively are shown in Table 1. Serum–virus mixtures were incubated for 2 hr. at room temperature (22° C.) followed by ½–1 hr. at 4° C. prior to inoculation into the test systems; results were read as before. The marked inhibition of cytopathogenicity by louping-ill hyperimmune sheep serum was regarded as proof of the specificity of the reaction.

HOLMAN E. WILLIAMS*

Animal Diseases Research Association,
Moredun Institute,
Gilmerton, Edinburgh.
Sept. 5.

* Present address: Department of Agriculture, Port of Spain, Colony of Trinidad and Tobago, British West Indies.

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monolayer tissue culture of pig kidney offers a feasible solution.

SUMMARY

1. Techniques are described for the preparation of monolayer tube-cultures of trypsin-dispersed pig, sheep, and chick embryo kidney tissues.

2. The virus of louping-ill multiplies in pig kidney tissue culture producing a cytopathogenic effect which is both readily visible and recognisable under low-power examination of the tube-culture. The effect is noticeable by the second to third day of incubation with 1 in 10 mouse brain virus (approximate titre of $10^{-7.7}$). Affected cells turn granular, the nuclei become swollen, and the cells appear to agglutinate. As cell necrosis increases the monolayer disintegrates.

3. Mouse brain virus is accurately titrated by its destructive action on pig cells and specific sheep anti-serum by its inhibitory action on virus cytopathogenicity. One CPD_{50} is equivalent to 300 to 600 mouse LD_{50}.

4. Virus does not appear to have any observable effect on cultures of sheep and chick embryo tissues examined in tubes under low-power magnification. It was not determined whether virus multiplication took place in these tissues.

CONCLUSION

While a satisfactory complement-fixation test has been developed for louping-ill disease in sheep, complement-fixing antibody is only of limited value in determining susceptibility, or immunity resulting from infection. Susceptible sheep are always negative to the test but those which recover from the disease are not always positive due to the delayed appearance, borderline titres, or rapid decline and disappearance of antibody. In contrast, serum neutralising antibody is a reliable diagnostic criterion. This antibody is stable in the recovered animal.

Louping-ill mouse brain virus is accurately titrated by its cytopathogenic effect in pig kidney monolayer tissue culture, and sheep antiserum by its inhibitory action on virus cytopathogenicity. Such titrations are as accurate as those in mice.

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